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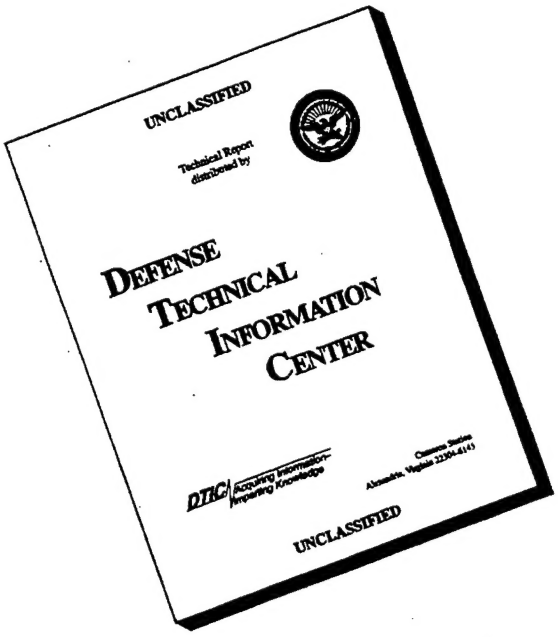

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Multiple methods have been used to evaluate the role of bacterial endotoxin(LPS) in the observed toxicities of solutions of hemoglobin(Hb). A series of physical measurements were performed to determine the effects of Hb on the LPS macromolecule. These included ultrafiltration, density centrifugation, precipitation, and gel filtration, as well as investigations specifically designed to evaluate binding of LPS by Hb and to determine whether complex formation occurs. The data support the conclusion that the interaction between LPS and Hb results in the formation of a complex. The formation of LPS-Hb complexes is associated with disaggregation of the LPS macromolecule and importantly, with marked enhancement of the biological activity of LPS. Enhancement of the biological activity of LPS has been shown in two independent biological systems, i.e., activation of the coagulation cascade of Limulus amebocyte lysate and stimulation of the production of tissue factor by human endothelial cells. Similar results have been shown for a wide variety of clinically relevant LPSs and for both purified, native HbA_0 and $\alpha\alpha$-crosslinked Hb. Therefore, enhancement of the biological activity of LPS is not limited to the crosslinked Hb that we selected for this initial group of experiments.

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Jack Lewis 9/15/95

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INTRODUCTION

The development of modified cell-free hemoglobin(Hb) as a blood substitute is of great importance in both military and civilian settings, and has been identified by the U.S. Army Medical Research, Development, Acquisition, and Logistics Command as a critical component of overall combat casualty care. Solutions of modified Hb have demonstrated adequate properties in regard to oxygen binding and delivery, in vivo half-life, lack of infectious risk, and in vitro stability for long-term storage. Therefore, a solution of Hb is a promising candidate for a red blood cell substitute for the emergency treatment of acute hemorrhage and probably for other clinical applications.

The principal limitation for the utilization of Hb for human use is in vivo toxicity, which has been demonstrated in numerous animal models. Although some of the data in earlier reports in this area, which described toxicity of Hb, may have resulted from the contamination of solutions of Hb with bacterial endotoxin(lipopolysaccharide, LPS) or erythrocyte stromal lipids, it is also possible that Hb possesses an intrinsic toxicity when administered as a solution.

The focus of our investigations has been the role of bacterial endotoxins(LPS) in the observed toxicities of solutions of Hb. LPS is a ubiquitous contaminant of the preparation of many protein solutions,

and probably has been the cause of some of the reported toxicity of Hb. However, in the course of our initial studies in this area, it became apparent that significant pathophysiologic consequences can result from a biochemical interaction(s) between Hb and LPS. Based on this background, the purpose of the present work is to characterize the nature of the interaction(s) between Hb and LPS, the effects of this interaction on the Hb and LPS molecules, and the pathophysiological effects that result.

Multiple methods have been used to approach this broad problem. A series of physical measurements have been performed to determine the effects of Hb on the LPS macromolecule. These include ultrafiltration, density centrifugation, precipitation, and gel filtration, as well as investigations specifically designed to evaluate binding of LPS by Hb and to determine whether complex formation occurs.

These in vitro studies have been complemented by the initiation of in vivo studies, the purpose of which is to characterize the distribution of endotoxin among both circulating blood cells and the major components of blood plasma, and to subsequently determine the potential effects of infusion of Hb upon the distribution of LPS.

SCIENTIFIC NARRATIVE*

In order to establish whether significant binding between Hb and LPS occurred, formal binding studies were performed by quantifying the binding of ^{125}I-labelled LPS to microtiter wells coated with Hb. Binding of LPS to Hb was shown to be concentration dependent and saturable(Figs. 1 and 2). The K_d was 3.1 x 10^{-8} M, a value which was closely approximated by calculation of the K_d based on sucrose centrifugation of LPS and Hb(6.3 x 10^{-8} M)(Table 1). Binding of LPS to Hb was more directly assessed using a derivatized LPS containing a photoactivatable group capable of covalently binding to proteins(Fig. 3). These key experiments demonstrated that LPS bound to both the alpha(α) and beta(β) chains of Hb, with greater affinity for Hb β chains.

Since earlier experiments using ultrafiltration had indicated that following incubation with Hb, LPS(which in aqueous solution behaves like a macromolecule) appeared to disaggregate and act like a molecule with M.W. less than 100,000 Kd, density gradient centrifugation and polyacrylamide gel electrophoresis were utilized to extend these observations. ^{14}C-labelled LPS was incubated with $\alpha\alpha$Hb(Hb cross-linked between α chains with bis(3,5-dibromo-salicyl)fumarate(DBBF) and then centrifuged through a 4-20% continuous sucrose gradient. LPS alone rapidly sedimented through this sucrose gradient, whereas in the presence of Hb, the

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* The data summarized in this section are provided in greater detail in the 9 published papers, attached to this Annual Report(see also REFERENCES).

sedimentation velocity was markedly slowed and the LPS co-migrated with the Hb(Fig. 4). In another experiment, ^{14}C-labelled LPS was incubated with $\alpha\alpha$Hb and then electrophoresed in the absence of SDS, so that LPS/Hb complexes remained intact. In the absence of $\alpha\alpha$Hb, all of the LPS remained in the stacking gel. In contrast, in the presence of Hb, from 23-45% of the LPS entered the gel and co-migrated with the Hb(Table 2). These data supported our hypothesis that Hb was capable of disaggregating LPS and forming stable LPS-Hb complexes. The data derived from filtration suggest that at least some LPS-Hb complexes are composed of a ratio of 2 LPS molecules: 1 Hb molecule.

The following series of experiments were performed to determine whether complex formation between LPS and Hb, which apparently produced disaggregation of LPS, was associated with alteration of the biological activity of LPS. We were able to document that in the presence of Hb, the biological activity of a wide variety of LPSs was increased, as determined by activation of the coagulation cascade of Limulus amebocyte lysate, i.e., the Limulus amebocyte lysate(LAL) test. The biological activity of both Proteus and Salmonella LPSs were increased in the presence of Hb(Figs. 5 & 6). This was true for both rough and smooth forms of LPS(Figs. 5 & 6). Furthermore, even deep rough(Re) LPS(S. minnesota 595) and purified Lipid A were enhanced, further establishing that the carbohydrate moiety of LPS was not required for the enhancement phenomenon. Singly dephos-phorylated MPL was enhanced less than the parent R 595 LPS whereas partially deacylated Re LPS(OH 37) was not enhanced by the

presence of LPS(Fig. 6). Therefore, the overall results suggested that phosphate and Kdo residues are less important in the enhancement process than the ester-linked fatty acids of Lipid A.

Additional physical and biological measurements were simultaneously performed to shed further insights into the nature of the interaction between LPS and Hb and its association with enhanced biological activity. Turbidity and activity in the LAL test were determined for a group of LPSs, and correlated with a series of concentrations of Hb. There was a dose/response relationship between the concentration of Hb, reduction of turbidity(as a measure of disaggregation) and increase of activity in the LAL test(as a measure of biological activity)(Fig. 7). Pertinently, S. minnesota MPL, which did not become disaggregated in the presence of Hb, did not demonstrate enhanced biological activity(Fig. 7). Additional experiments demonstrated that enhancement of LPS activity by Hb was not dependent upon the salt form of the LPS(Table 3). Non-toxic LPSs derived from Rhodobacter species were not enhanced by Hb(Table 3). Other evidence of enhancement was provided by studies which demonstrated that the limit of detection of LPS was lowered 10-fold in the presence of Hb(Table 4).

Although the above series of experiments appeared to clearly document that the interaction between Hb and LPS resulted in enhancement of the biological activity of LPS, it was necessary to establish that this phenomenon was not limited to a single assay system. Therefore, another group of experiments were performed to

determine the effects of this interaction on the production of the procoagulant tissue factor (TF) by human endothelial cells. A culture system of HUVEC(human umbilical vein endothelial cells) was chosen as another assay for the biological activity of LPS because endothelial cells(EC), which normally do not produce TF, can be stimulated by LPS to produce this important procoagulant activity. In the presence of Hb, which by itself did not stimulate the production of TF by EC, a variety of LPSs generated greater amounts of TF activity than did identical concentrations of LPS in the absence of HB(Figs. 8 & 9). Furthermore, the enhancement of production of TF by EC in the presence of LPS and Hb was confirmed using an immunological assay for TF protein. It was also shown that protein production was required for this effect, since TF production was blocked by either cycloheximide or actinomycin D(data not shown).

The ability of other proteins to enhance the biological activity in the LAL test was evaluated. LBP(lipopolysaccharide binding protein) was shown to more potently enhance the biological activity of LPS than did Hb(Fig. 10). However, human serum albumin was less effective than Hb, and IgG and transferrin failed to enhance the biological activity(Fig. 11). This series of experiments demonstrated that the effect of Hb on LPS was not a non-specific effect of protein.

The next major goal of our investigations during the past year was to initiate studies of the effects of LPS on Hb, to determine if the interaction between these molecules altered both of the components of the LPS-Hb complex. Experiments were performed to measure the

production of free radicals by solutions of Hb, utilizing the FOX reagent. Although there was Hb concentration-dependent production of free radicals, the addition of LPS did not increase the amount of free radicals produced(Fig. 12).

Oxygen equilibrium curves for Hb in the absence and presence of Hb were then determined. An equilibrium curve in the absence of LPS was established for ααHb(Fig. 13). In the presence of LPS, the oxygen equilibrium curve was unchanged(Fig. 14). Other controls were established with hemolyzed normal blood(Fig. 15) and with non-crosslinked HbA_0(Fig. 16). In contrast to the lack of effect of LPS on the equilibrium curve for crosslinked ααHb, LPS did slightly shift the curve when incubated with HbA_0. In this instance, oxygen affinity was increased(Fig. 17). The effect of LPS on non-crosslinked HbA_0 was also demonstrated with singly deacylated S. minnesota 505 LPS, documenting that this effect was not limited to a single LPS(Fig. 18). A final group of observations were made to directly compare the effects of LPS on ααHb and HBA_0, Both smooth and rough LPSs were utilized(Table 5). In this series of experiments, ααHb demonstrated only a slight trend to higher oxygen affinity, whereas HbA_0 demonstrated a more marked increase in oxygen affinity.

Preliminary experiments had suggested that LPS produced circular dichroic(CD) spectral changes in Hb. In order to further characterize these changes, formal CD spectra were obtained of Hb, in the presence and absence of LPS. In the presence of LPS, the CD spectrum of Hb demonstrated decreased intensities of the near

UV(259 and 265 nm), Soret(420 nm) and visible peaks(545 and 579 nm); and a shift in the Soret peak maximum from 420 nm to 418 nm. These spectral changes were consistent with production of metHb, without substantial changes in the protein secondary structure(Fig. 19). α-helical content was estaimated to be 53% for the globin, both in the absence and presence of LPS. Detailed calculations of measurements of the protein secondary structure of ααHb in the absence and presence of Hb are summarized in Table 6.

The above in vitro experiments were complemented by initial in vivo experiments to determine the effects of Hb on the clearance and distribution of LPS in rabbit. These experiments were undertaken to begin evaluation of the potential physiological significance of the interaction between LPS and Hb that we had demonstrated in vitro. The relevance of these studies was increased by the likelihood that some of the recipients of hemoglobin solutions would have concomitant endotoxemia or would develop endotoxemia in association with trauma, sepsis, hypotension, or translocation of LPS from the GI tract into the portal circulation. Initial experiments revealed that the infusion of Hb delayed the intravascular clearance of ^{125}I-LPS(Fig. 20), but that there was no additional decrease in white blood cells or platelets when Hb was administered shortly after the i.v. administration of LPS.

CONCLUSIONS

The data summarized in the Scientific Narrative support the conclusion that the interaction between bacterial endotoxin(LPS) and hemoglobin(Hb) results in the formation of a complex. The formation of LPS-Hb complexes is associated with disaggregation of the LPS macromolecule and importantly, with marked enhancement of the biological activity of LPS. Importantly, enhancement of the biological activity of LPS has been shown in two independent biological systems, i.e., activation of the coagulation cascade of Limulus amebocyte lysate and stimulation of the production of tissue factor by human endothelial cells. These results have been shown for a wide variety of clinically relevant LPSs and for both purified, native HbA_0 and $\alpha\alpha$ crosslinked Hb. Therefore, the enhancement of the biological activity of LPS is not limited to the crosslinked Hb that we selected for this initial group of experiments.

Since solutions of Hb are likely to be administered to patients who have suffered trauma, are hypotensive, or are septic, it follows that many of the recipients of Hb, will either have concomitant endotoxemia or will develop endotoxemia. Liver disease and damage to the gastrointestinal tract are also associated with endotoxemia. Therefore, the possibility that the pathophysiological effects of LPS will be enhanced in the presence of circulating free Hb must be seriously considered and the potential clinical consequences anticipated and evaluated.

In addition, it is likely that complex formation will also affect Hb, and therefore, thorough investigation of the effects of LPS-Hb complex formation on the hemoglobin molecule is required. Accordingly, we plan to continue studies of the effects of the interaction between LPS and Hb on the Hb molecule. These studies should be complemented by an attempt to characterize the biochemical nature of the interaction between these two molecules.

In addition, the effects of the administration of both LPS and Hb will be investigated in an animal model, with the goal of determining if the pathological effects of LPS are increased in the presence of Hb. If such an effect is detected, we will then seek to determine the mechanism by which increased mortality is produced.

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FIGURE LEGENDS

Fig. 1. Experiment: Preliminary binding studies were performed to characterize the interaction of LPS with Hb. ααHb (1 μg/well) was immobilized in microtiter plate wells, and various concentrations of ^{125}I-LPS were added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb.

Conclusion: Concentration-dependent binding of LPS to Hb was demonstrated.

Fig. 2. Experiment: Based on the preliminary binding results (above), additional studies were performed to more completely characterize the interaction of LPS with Hb. ααHb (1 μg/well) was immobilized in microtiter plate wells, and various quantities of ^{125}I-LPS were added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb.

Conclusion: Saturable binding of LPS to Hb was demonstrated.

Fig. 3. Experiment: Direct binding of LPS to Hb was assessed using a derivatized LPS containing a photoactivatable group capable of covalently binding to proteins. This derivatized LPS (S. minnesota Re595 LPS-(p-azidosalicylamido)-1,3'-dithio-proprionamide)(^{125}I-LPS-ASD) also was radioiodinated in order to detect its binding to protein. ^{125}I-LPS-ASD was incubated with ααHb, photolyzed with UV light, and electrophoresed in SDS and 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie blue (A), dried, and subjected to autoradiography (B, left lane). Controls consisted of an incubation mixture containing 100-fold excess unlabeled LPS as a blocking agent to demonstrate inhibition of specific binding (B, middle lane) and ^{125}I-LPS-ASD alone (B, right lane).

Conclusion: Specific binding of the photoaffinity labeled LPS to Hb was demonstrated, with greater affinity for Hb β chains.

Fig. 4. Experiment: Binding of LPS (which rapidly sediments in sucrose) to Hb (which slowly sediments) was analyzed by density gradient centrifugation. ^{14}C-LPS was incubated with $\alpha\alpha$Hb (100 mg/ml), and the mixture centrifuged through a 4-20% continuous sucrose gradient. 0.4 ml fractions were assayed for hemoglobin by absorbance at 405 nm (closed symbols), and for LPS by scintillation counting (open symbols).

Conclusion: LPS and Hb co-migrated, with a sedimentation velocity less than that of LPS alone.

Fig. 5. Experiment: Enhancement by Hb of the biological activity of a variety of Proteus LPSs (each at 500 ng/ml) was determined by comparison of LPS activities in the chromogenic LAL test in the absence (-Hb) or presence (+Hb) of Hb (1 mg/ml). To determine relative LAL activities, a standard curve of P. mirabilis R45 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R45 LPS concentration. 500 ng/ml R45 LPS was assigned a relative LAL activity of 1. The fold increase in LAL activity of each LPS, induced by Hb, is indicated in parentheses. Samples were assayed with eight replicates, and results are expressed as the mean $\pm$ 1 S.D.
LPSs studied: smooth (S1959 and 03 LPS); rough Ra (R110); rough Re (R45)

Conclusion: The biological activities of Proteus LPSs were dramatically enhanced in the presence of Hb.

Fig. 6. Experiment: Enhancement by Hb of the biological activity of a variety of Salmonella LPSs (each at 500 ng/ml) was determined by comparison of LPS activities in the chromogenic LAL test in the absence (-Hb) or presence (+Hb) of Hb (1 mg/ml). To determine relative LAL activities, a standard curve of parent S. minnesota Re 595 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent Re 595 LPS concentration. 500 ng/ml Re 595 LPS was assigned a relative LAL activity of 1. The fold increase in LAL activity of each LPS, induced by Hb, is indicated in parentheses. Samples were assayed with eight replicates, and results are expressed as the mean $\pm$ 1 S.D.
LPSs studied: native Re LPS (595); singly deacylated 595 LPS (OH37); multiply deacylated 595 LPS (OH56); lipid A; monophosphoryl lipid A (MPL)

Conclusion: The biological activity of S. minnesota 595 LPS was enhanced in the presence of Hb. Lipid A and the other LPS partial structures were enhanced by Hb to a much lesser extent or not at all.

Fig. 7. Experiment: To determine the relationship between Hb-induced enhancement of LPS biological activity and LPS disaggregation, turbidity and biologic activities of LPSs were determined in the absence and presence of Hb. Various concentrations of ααHb (from 0.01 to 1.0 mg/ml) were added to LPS (final concentration, 1 mg/ml) in microtiter plate wells and absorbances were measured at 620 nm. The turbidity of each LPS (absorbance at 620 nm) in the absence of Hb has been designated as 0, and the change in absorbance induced by Hb is shown. Absorbances due to Hb have been subtracted. Actual baseline LPS absorbances were as follows: P. mirabilis R110, 0.21; S. minnesota R 595, 0.12; S. minnesota lipid A, 0.61; and S. minnesota MPL, 0.65. LAL then was added to each well and chromogenic activity determined at 405 nm.

Conclusion: Hb-induced enhancement of P. mirabilis R110 LPS, S. minnesota 595 LPS and, to a lesser extent, S. minnesota lipid A was associated with a concomitant decrease in LPS aggregation state. S. minnesota MPL, which did not become disaggregated in the presence of Hb, did not demonstrate enhanced biological activity.

Fig. 8. Experiment: Enhancement by Hb of the biological activity of a variety of Proteus LPSs (each at 10 μg/ml) was determined by comparison of tissue factor production by cultured human umbilical vein endothelial cells in the absence (-Hb) or presence (+Hb) of Hb (10 mg/ml). Tissue factor (TF) activities were determined with a plasma recalcification assay. The fold increase in TF induced by Hb for each LPS is indicated in parentheses. The means of 4 wells are presented.
LPSs studied: smooth (S1959 and 03 LPS); rough Ra (R110); rough Re (R45)

Conclusion: The biological activities of several Proteus LPSs were dramatically enhanced in the presence of Hb.

Fig. 9. Experiment: Enhancement by Hb of the biological activity of a variety of Salmonella LPSs (each at 10 μg/ml) was determined by comparison of tissue factor production by cultured human umbilical vein endothelial cells in the absence (-Hb) or presence (+Hb) of Hb (10 mg/ml). Tissue factor (TF) activities were determined with a plasma recalcification assay. The fold increase in TF induced by Hb for each LPS is indicated in parentheses. The means of 4 wells are presented.
LPSs studied: native Re LPS (595); multiply deacylated 595 LPS (OH56); monophosphoryl lipid A (MPL).

Conclusion: Tissue factor production induced by 595 LPS and lipid A was dramatically enhanced by Hb. The biological activity of multiply deacylated OH56 LPS was unaffected by Hb.

Fig. 10. Experiment: Hb and lipopolysaccharide binding protein (LBP) were compared for their relative abilities to enhance LPS activation of LAL. S. minnesota Re 595 LPS (10 pg/ml), in the absence or presence of $\alpha\alpha$Hb (in concentrations ranging from 1-100 μg/ml) or LBP (in concentrations ranging from 0.01-10 μg/ml), was assayed with the chromogenic LAL test. Samples were assayed in triplicate, and results are expressed as the mean ± 1 S.D.

Conclusion: LPS biological activity was increased in the presence of both LPB and Hb. Throughout almost the entire range of Hb concentrations tested, equivalent enhancement of LPS activity was produced by LBP at a much lower protein concentration.

Fig. 11. Experiment: In order to assess the extent to which enhancement of LPS biological activity is specific for Hb, the influence of a variety of plasma LPS-binding proteins on the ability of S. minnesota Re 595 LPS to activate LAL was examined. S. minnesota Re 595 LPS (30 ng/ml) was incubated for 5 min at 37°C with native hemoglobin (HbA_0), human albumin (HSA), human immunoglobulin (IgG), or human transferrin (Tf) (each at concentrations from 0.015 to 0.5 mg/ml), and chromogenic LAL assays were performed. Absorbance at 405 nm for LPS alone was 0.24. Samples were assayed in duplicate, and mean values are presented.

Conclusion: Both Hb and HSA enhanced the biological activity of LPS in a dose-dependent manner, although the enhancement effect of Hb was detectable at 0.03 mg/ml whereas equivalent enhancement by HSA was only observed at >0.25 mg/ml. Neither IgG nor transferrin had any effect on LPS biological activity.

Fig. 12. Experiment: In order to assess the potential for LPS-induced production of free radicals by Hb, $\alpha\alpha$Hb (0.05 to 1 mg/ml) was incubated at 37°C for 60 min in the absence (■) or presence (●) of 1 mg/ml OH37 LPS. FOX reagent was then added, and oxidizing products were detected by absorbance at 570 nm. LPS alone (1 mg/ml) (Δ) did not produce detectable oxidants. Each point is the mean of two determinations. The data are representative of three independent experiments.

Conclusion: Hb concentration-dependent production of free radicals was demonstrated. This process was not affected by the addition of LPS.

Fig. 13. Experiment: The oxygen equilibrium curve for normal blood was determined with a Hemox-analyzer.

Conclusion: A normal sigmoid curve was obtained.

Fig. 14. Experiment: The oxygen equilibrium curve for ααHb, in the absence and presence of LPS, was determined with a Hemox-analyzer. Hb (10 mg/ml) was analyzed alone, with 1 mg/ml P. mirabilis 03 LPS or with 1 mg/ml S. minnesota 595 LPS.

Conclusion: A normal sigmoid curve was obtained for ααHb alone. In the presence of LPSs, the Hb oxygen affinity curves were essentially unchanged.

Fig. 15. Experiment: The oxygen equilibrium curve for hemolyzed normal blood was determined with a Hemox-analyzer.

Conclusion: The oxygen equilibrium curve for hemolyzed blood was shifted far to the left (higher affinity), compared to Hb within intact erythrocytes (Fig. 2, above).

Fig. 16. Experiment: The oxygen equilibrium curve for non-crosslinked HbA_0 was determined with a Hemox-analyzer.

Conclusion: The saturation curve for HbA_0 was shifted far to the left (higher affinity) compared to crosslinked ααHb (Fig. 3, above).

Fig. 17. Experiment: The oxygen equilibrium curve for non-crosslinked HbA_0 (10 mg/ml), in the presence of smooth E. coli 026 LPS (1 mg/ml), was determined with a Hemox-analyzer.

Conclusion: In the presence of E. coli 026 LPS, HbAo oxygen affinity was slightly increased compared to HbA_0 alone (Fig. 5, above).

Fig. 18. Experiment: The oxygen equilibrium curve for non-crosslinked HbA_0 (10 mg/ml), in the presence of singly deacylated rough S. minnesota 595 LPS (OH37, 1 mg/ml), was determined with a Hemox-analyzer.

Conclusion: In the presence of OH37 LPS, HbA_0 oxygen affinity was slightly increased compared to HbA_0 alone (above).

Fig. 19. Experiment: Preliminary experiments had suggested that LPS induced circular dichroic (CD) spectral changes in Hb consistent with the formation of oxidized Hb species. In order to further characterize these changes, additional CD spectra of Hb were obtained , in the presence and absence of LPS, and of metHb. CD spectra were measured at room temperature between 200 nm and 600 nm for ααHb alone (13.8 μM heme, ambient oxygenation), ααHb/LPS (13.8 μM heme, 0.5 mg/ml S. minnesota OH37 LPS, obtained after 2-hr incubation at 37°C), and metHb (31.5 μM heme). Measurements in the far UV region were made with samples diluted 5 to 10-fold. A 1-cm pathlength cell was utilized for measurements between 250-400 nm and 430-600 nm, and a 0.2-cm pathlength cell was utilized for measurements of the major Soret (400-440 nm) and far UV (210-250 nm) regions. Ellipticities [θ] are expressed on a molar heme basis. Wavelengths for the Soret peak maxima are identified on inset tracings presented with an expanded X-axis.

Conclusion: In the presence of S. minnesota OH37 LPS, the CD spectrum of Hb demonstrated decreased intensities of the near UV (259 and 265 nm), Soret (420 nm) and visible peaks (545 and 579 nm), and a shift in the Soret peak maximum shifted from 420 nm to 418 nm. These spectral changes are consistent with production of a substantial quantity of metHb, without substantial changes in protein secondary structure. α-helical content was estimated to be 53% for the globin in both the absence and presence of LPS. (See Table 6).

Fig. 20. Experiment: The effect of Hb infusion on the intravascular clearance of ^{125}I-LPS in rabbits was examined. Rabbits initially were infused with either ααHb (□, n=5) or human serum albumin (HSA) (Δ, n=6)) equal to 25% of their blood volume, or received no infusion (O, n=6). All rabbits then were injected intravenously with ^{125}I-LPS (13 μg/kg) and serial blood samples obtained to determine LPS clearance. Values represent the mean (± SD) percent of the level of radioactive LPS in whole blood at T_0.

Conclusion: LPS clearance in rabbits which received Hb was significantly delayed compared to control rabbits (p=0.0007) or those which received HSA (p=0.03).

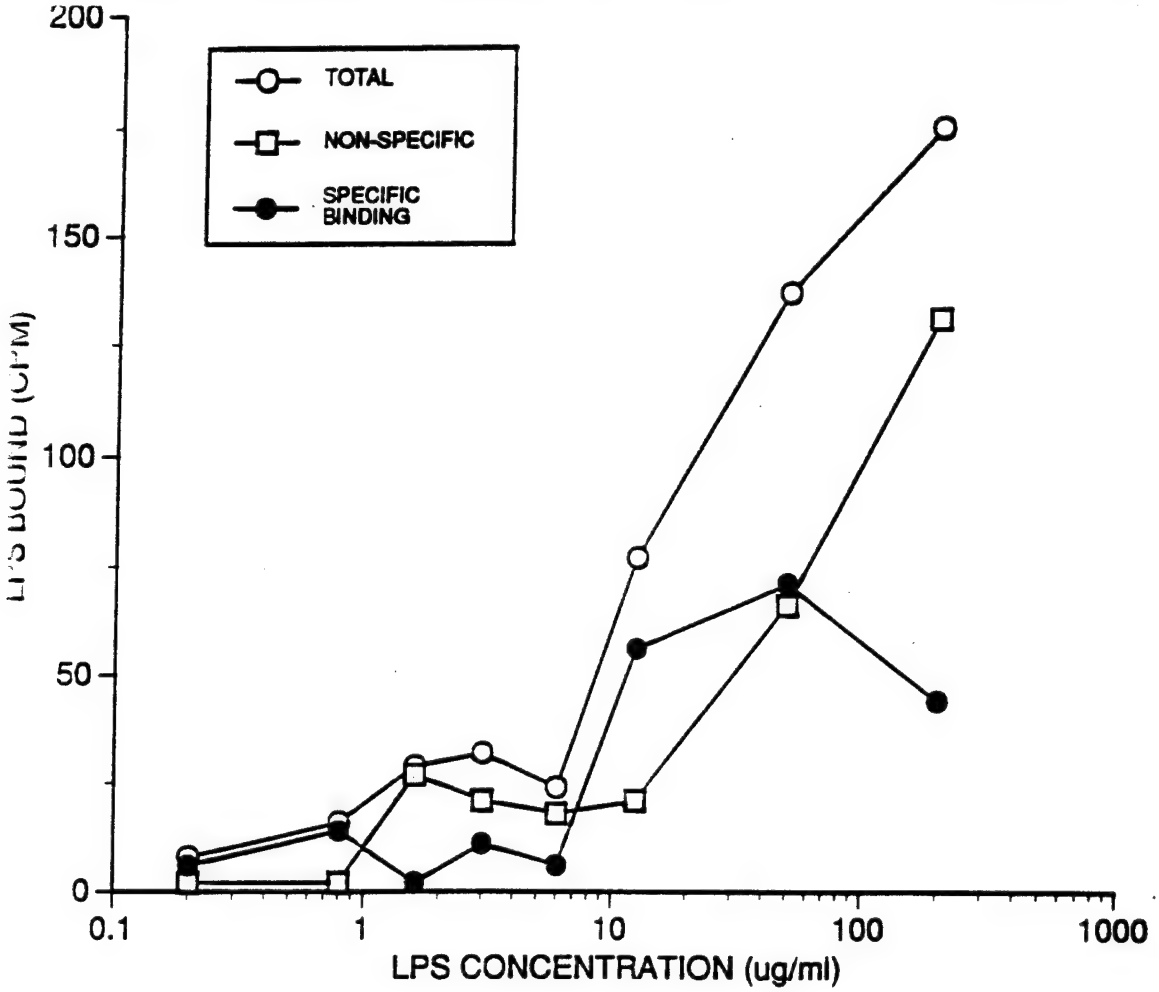

TOTAL
NON-SPECIFIC
SPECIFIC BINDING
200
150
100
50
0
0.1
1
10
100
1000
LPS CONCENTRATION (ug/ml)

Fig. 1

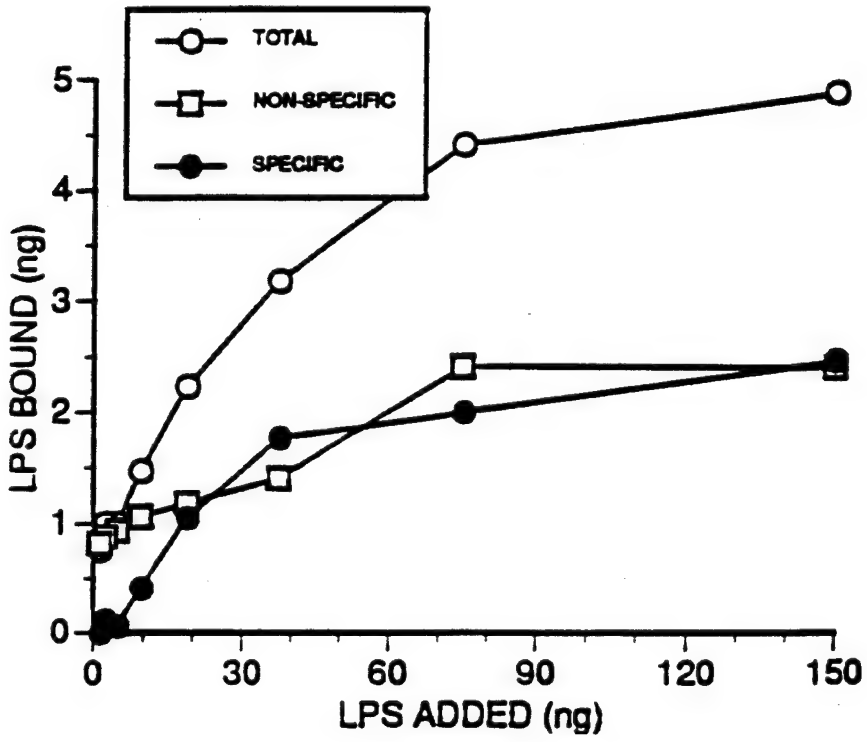

TOTAL
NON-SPECIFIC
SPECIFIC
LPS BOUND (ng)
5
4
3
2
1
0
0
30
60
90
120
150
LPS ADDED (ng)

Fig. 2

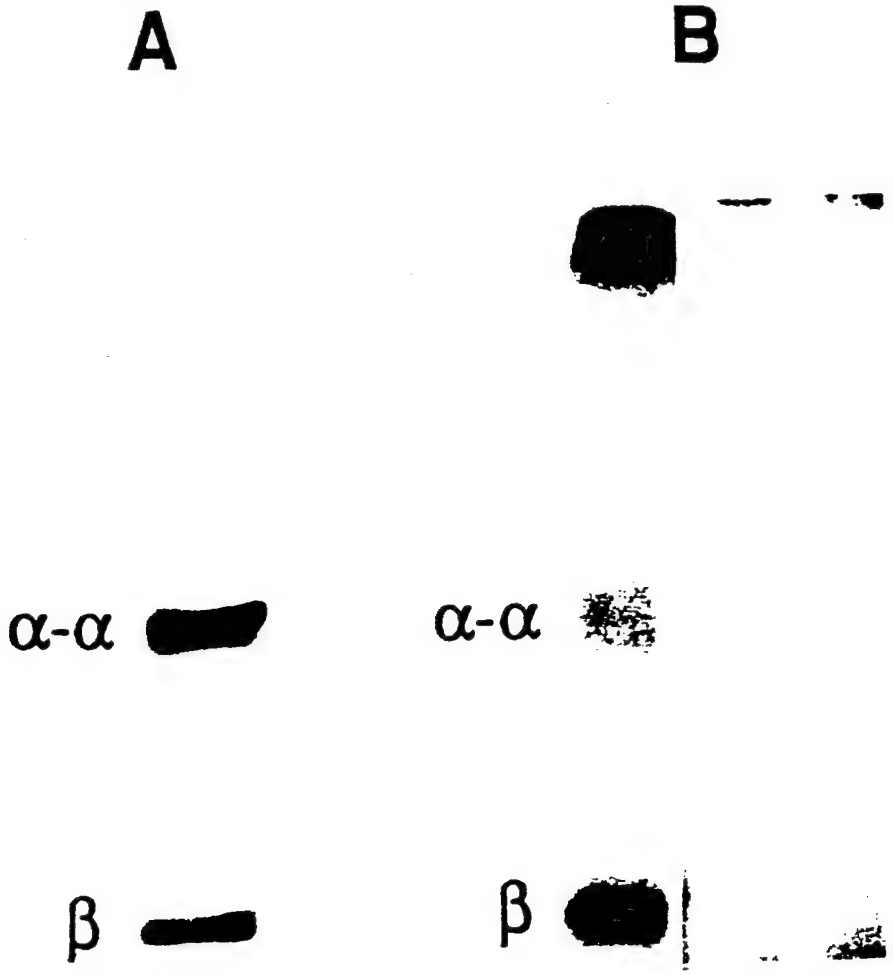

A
B
α-α
α-α
β
β

Fig. 3

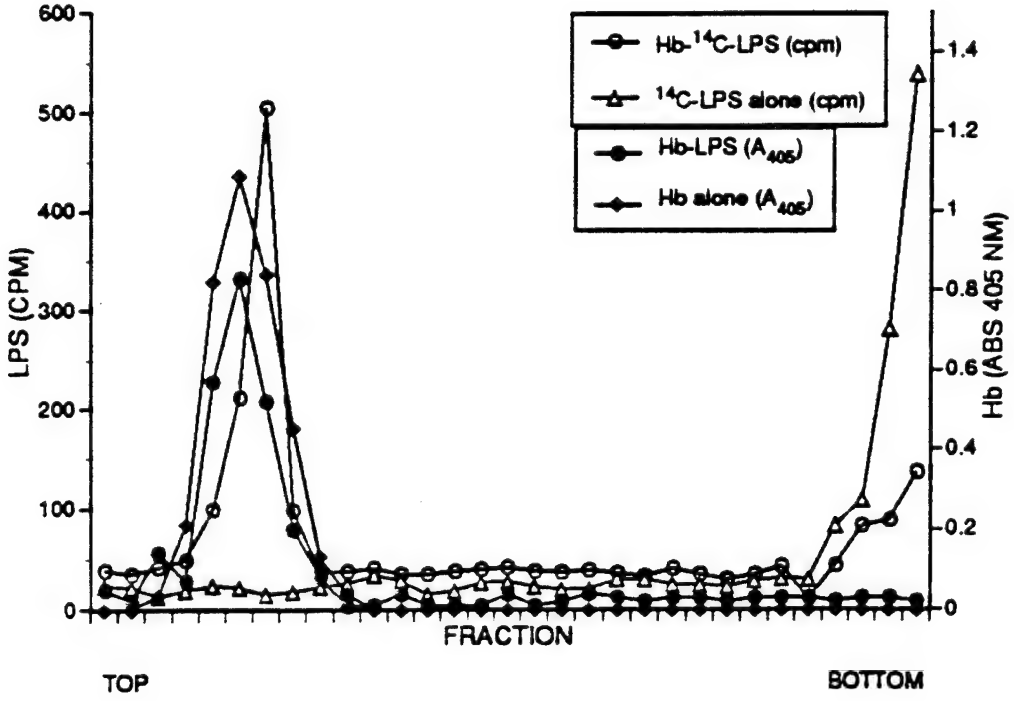

Hb-14C-LPS (cpm)
14C-LPS alone (cpm)
Hb-LPS (A405)
Hb alone (A405)
LPS (CPM)
Hb (ABS 405 NM)
FRACTION
TOP
BOTTOM
600
500
400
300
200
100
0
1.4
1.2
1
0.8
0.6
0.4
0.2
0

Fig. 4

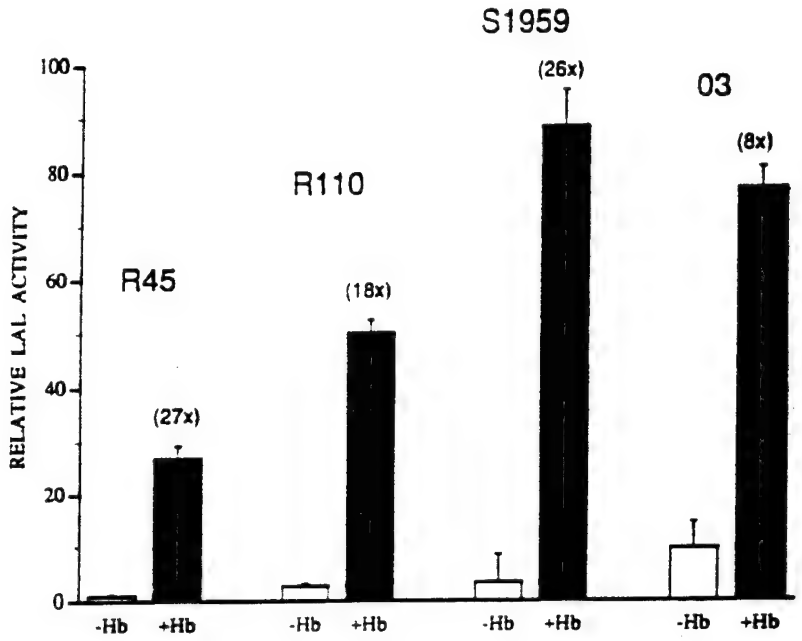

S1959
RELATIVE LAL ACTIVITY
100
80
60
40
20
0
R45
R110
03
(27x)
(18x)
(26x)
(8x)
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb

Fig. 5

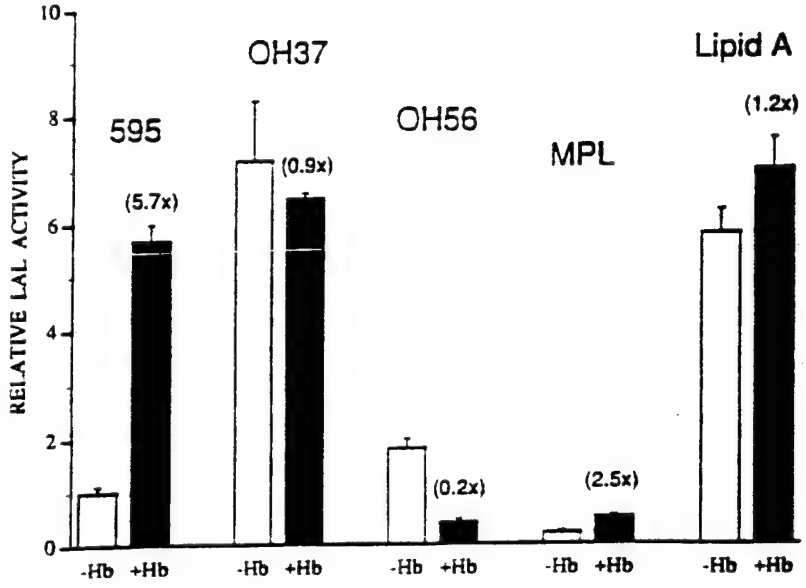

RELATIVE LAL ACTIVITY
10
8
6
4
2
0
595
OH37
OH56
MPL
Lipid A
(5.7x)
(0.9x)
(0.2x)
(2.5x)
(1.2x)
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb

Fig. 6

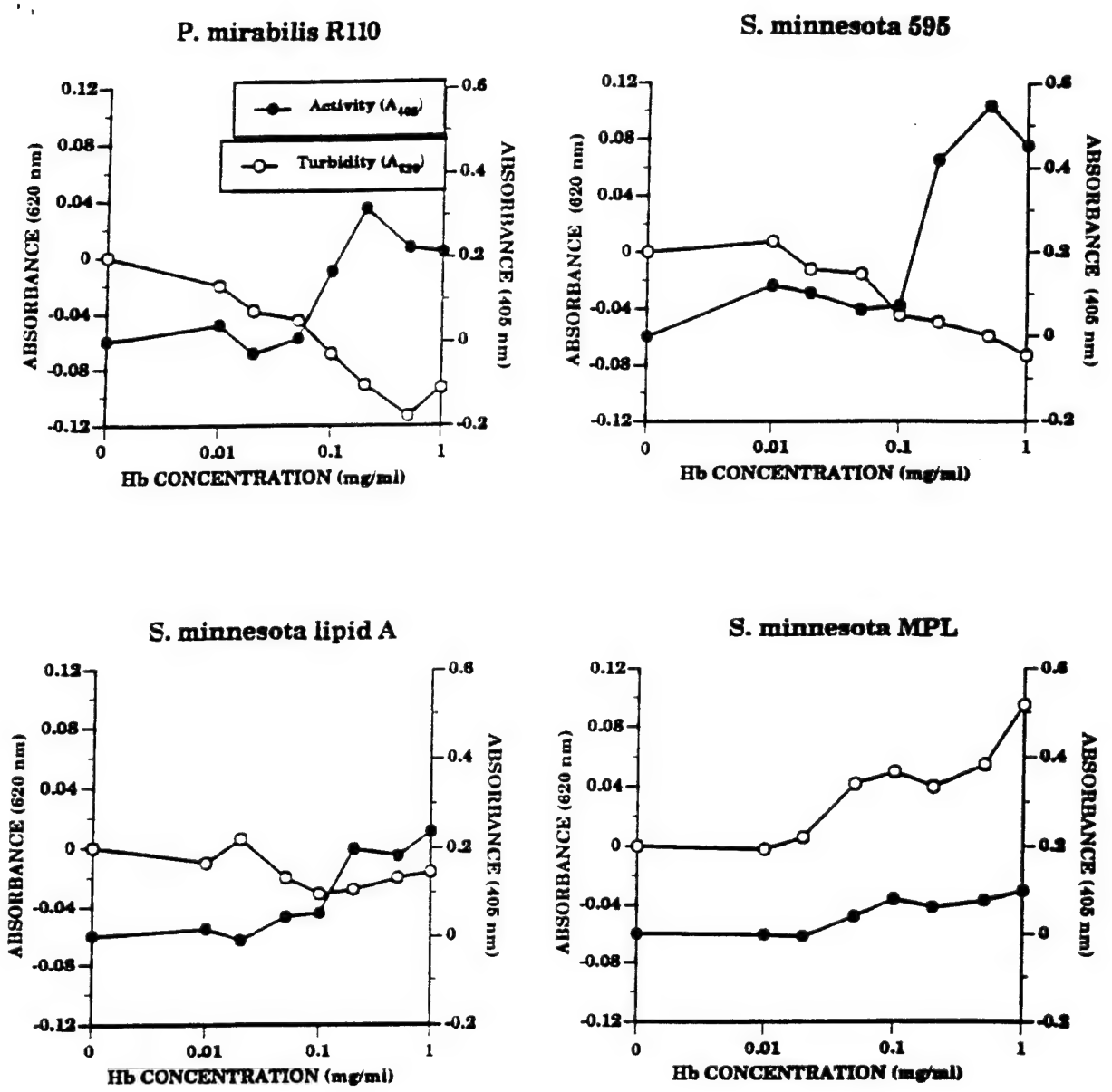

P. mirabilis R110
S. minnesota 595
S. minnesota lipid A
S. minnesota MPL
Activity (A_{405})
Turbidity (A_{620})
ABSORBANCE (620 nm)
ABSORBANCE (405 nm)
Hb CONCENTRATION (mg/ml)
0.12
0.08
0.04
0
-0.04
-0.08
-0.12
0.6
0.4
0.2
0
-0.2
0
0.01
0.1
1

Fig. 7

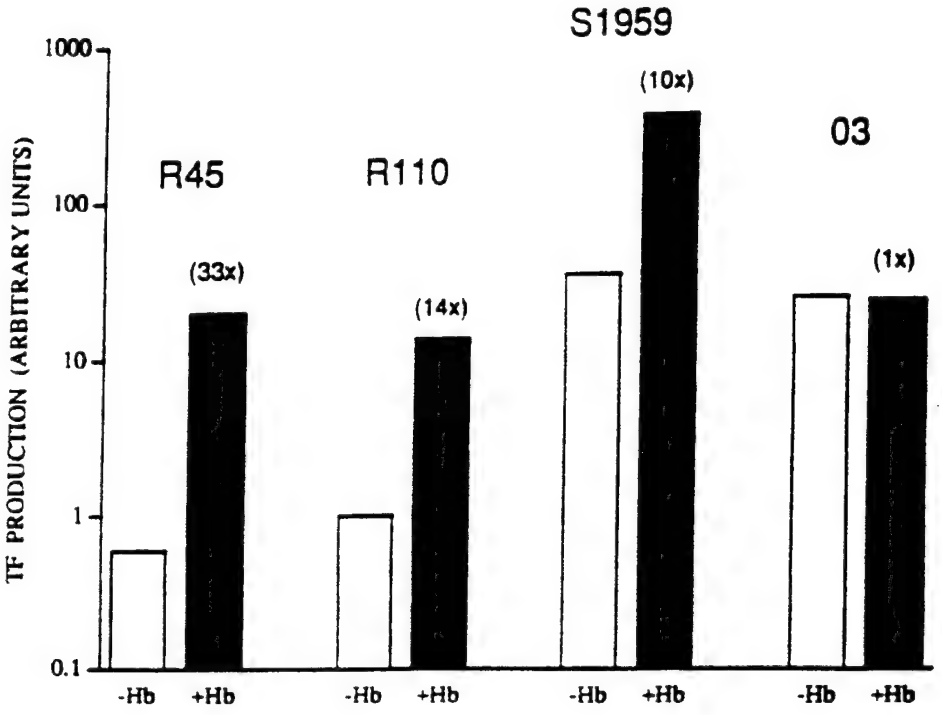

S1959
1000
100
10
1
0.1
TF PRODUCTION (ARBITRARY UNITS)
R45
R110
03
(33x)
(14x)
(10x)
(1x)
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb

Fig. 8

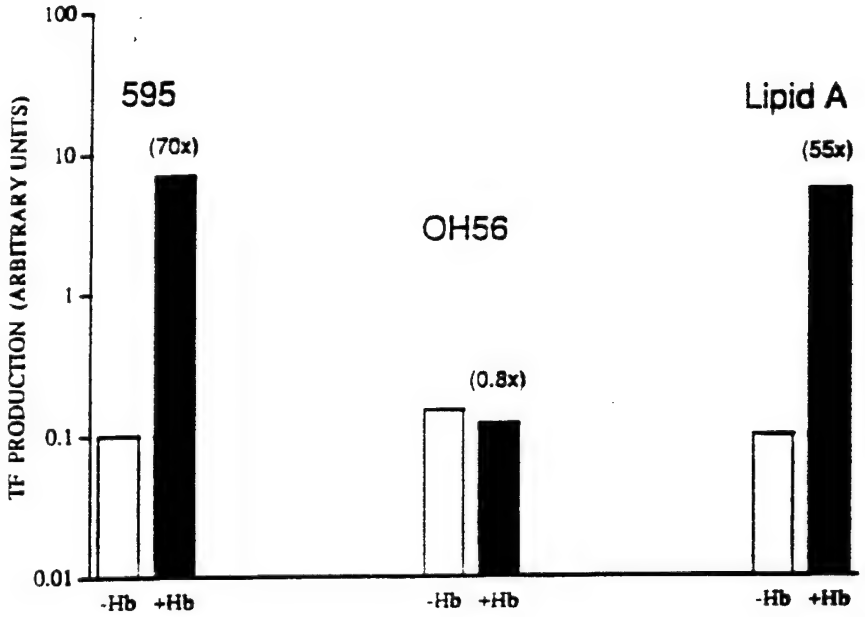

100
10
1
0.1
0.01
TF PRODUCTION (ARBITRARY UNITS)
595
OH56
Lipid A
(70x)
(0.8x)
(55x)
-Hb
+Hb
-Hb
+Hb
-Hb
+Hb

Fig. 9

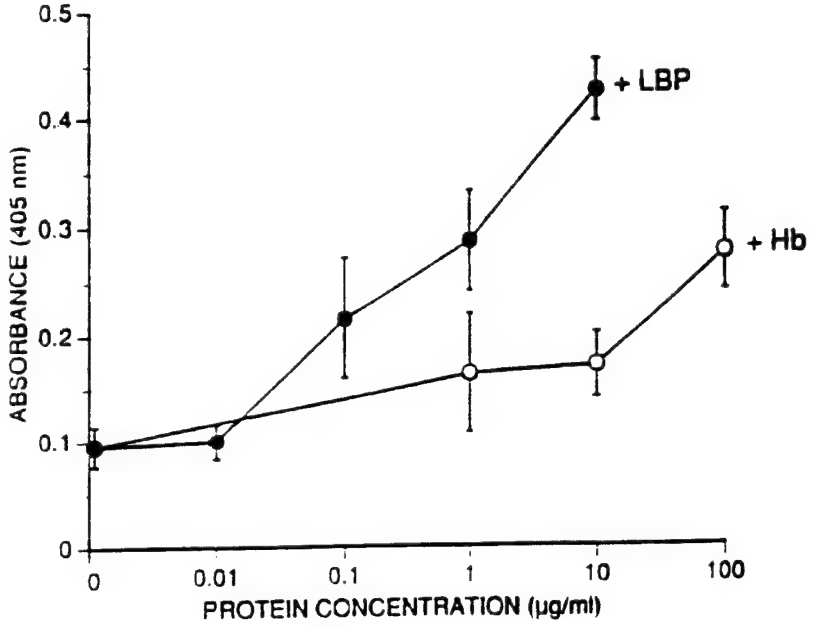

0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (405 nm)
+ LBP
+ Hb
0
0.01
0.1
1
10
100
PROTEIN CONCENTRATION (µg/ml)

Fig. 10

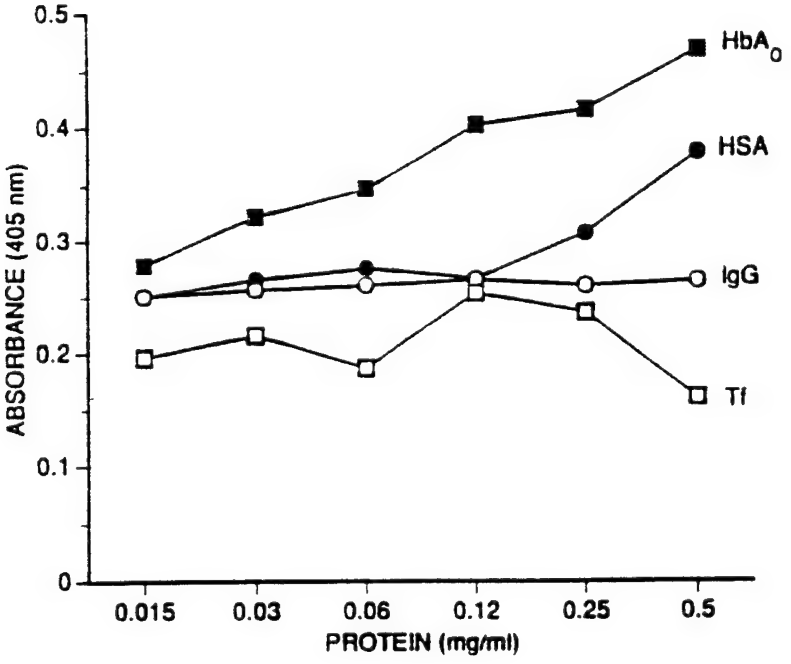

0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (405 nm)
HbA0
HSA
IgG
Tf
0.015
0.03
0.06
0.12
0.25
0.5
PROTEIN (mg/ml)

Fig. 11

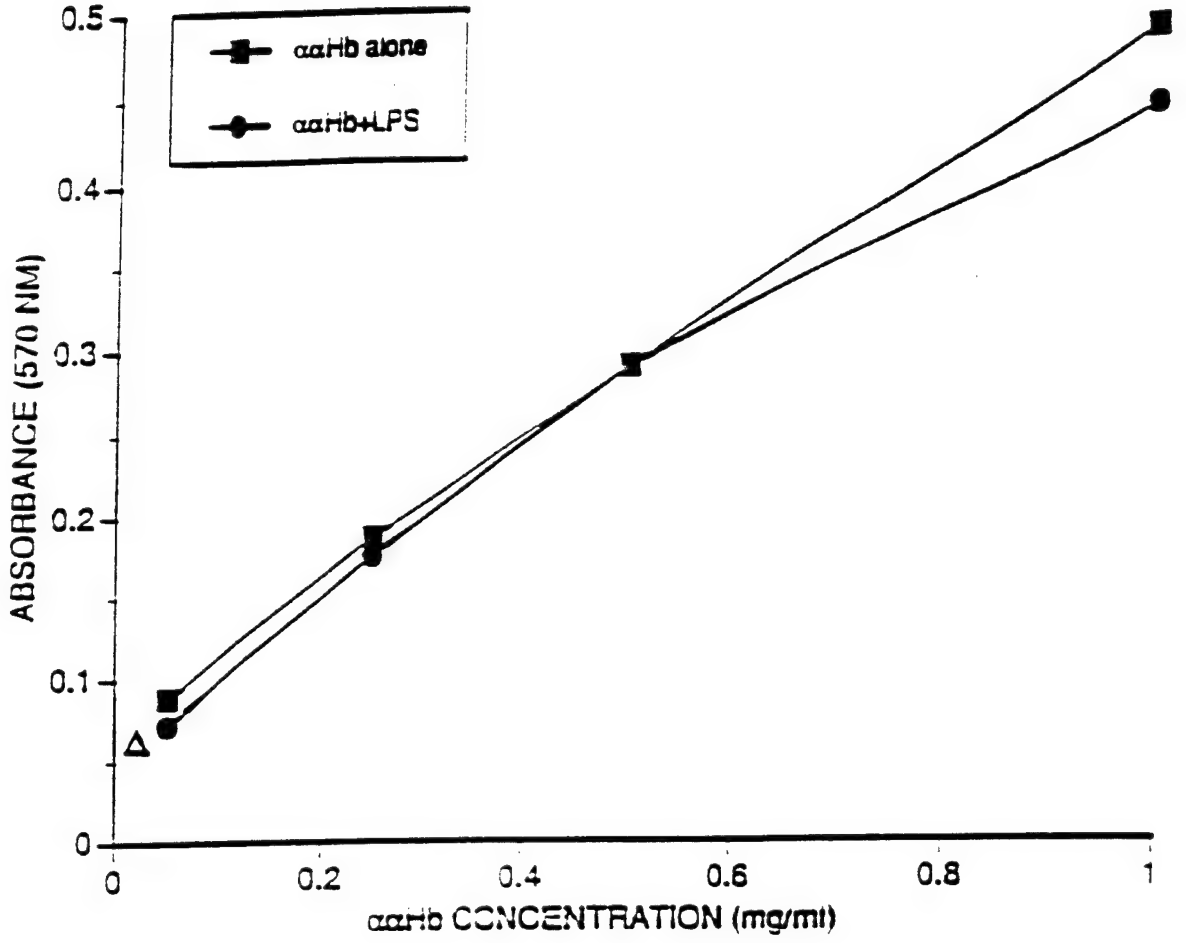

αα-Hb alone
αα-Hb+LPS
ABSORBANCE (570 NM)
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
αα-Hb CONCENTRATION (mg/ml)

Fig. 12

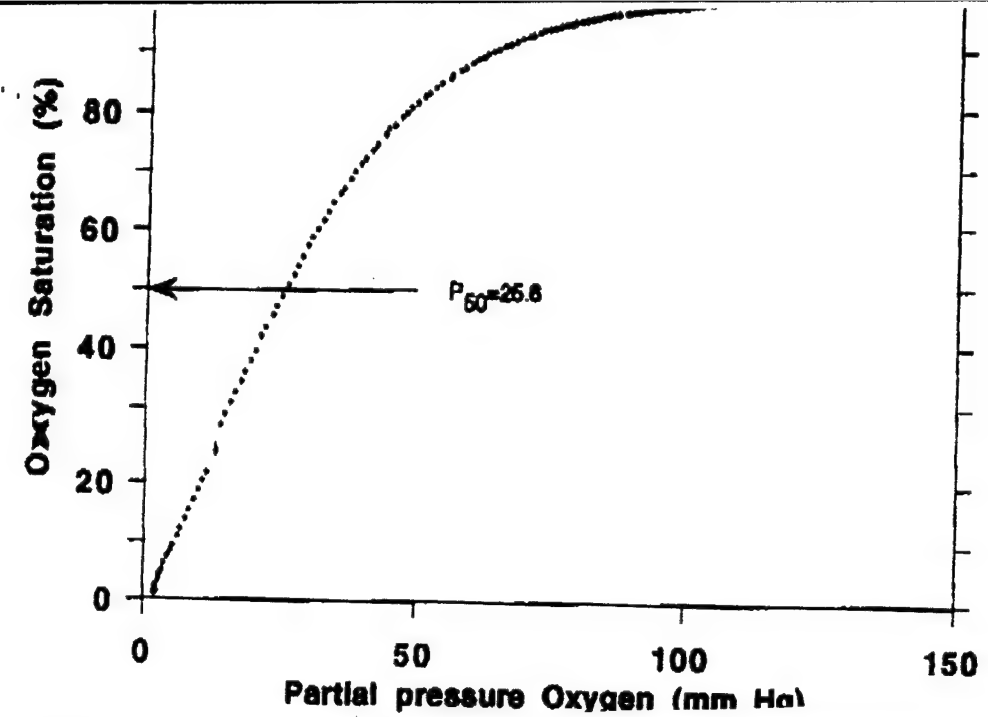


Fig. 13

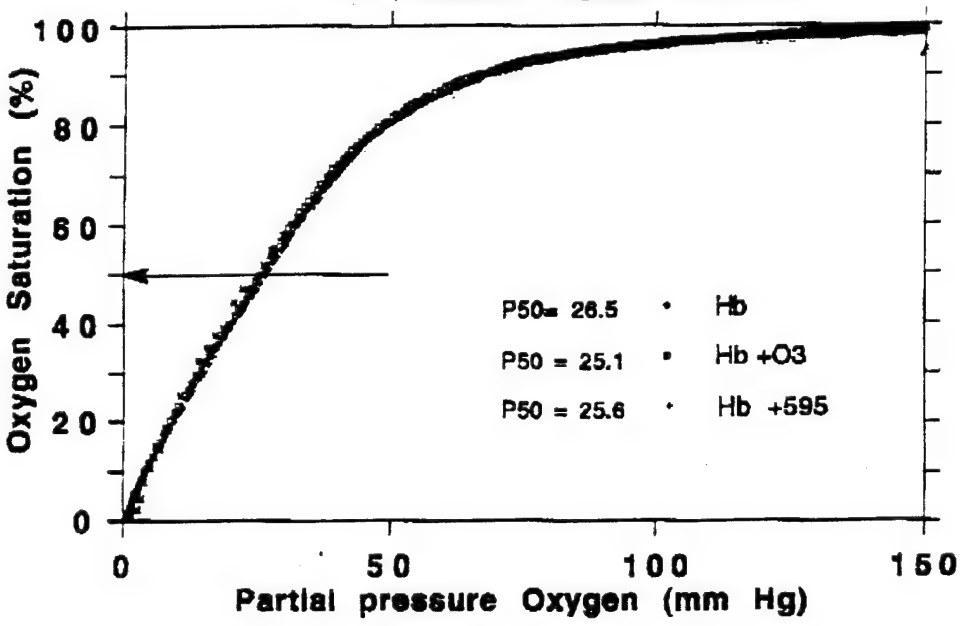


Fig. 14

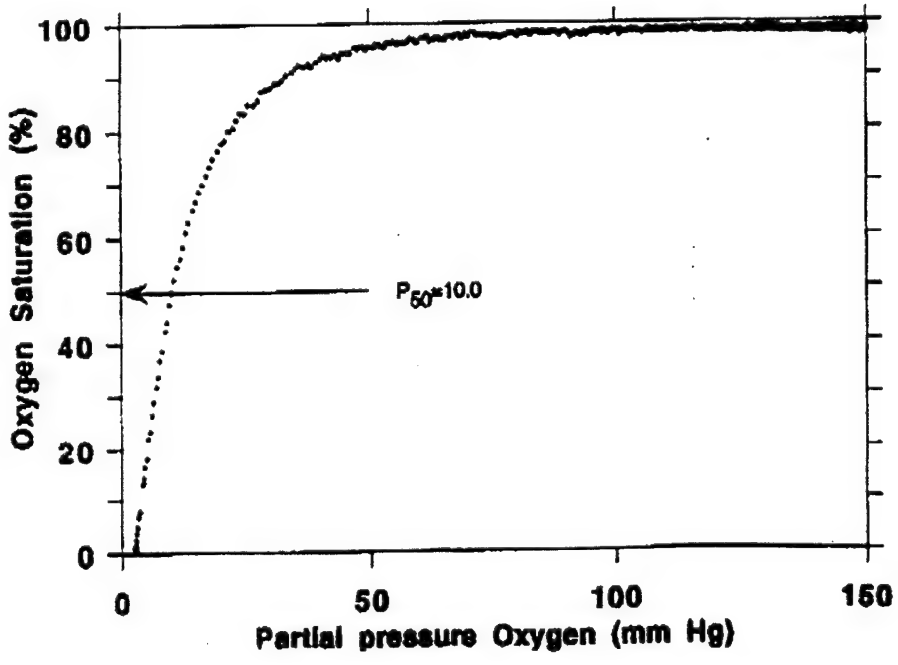


Fig. 15

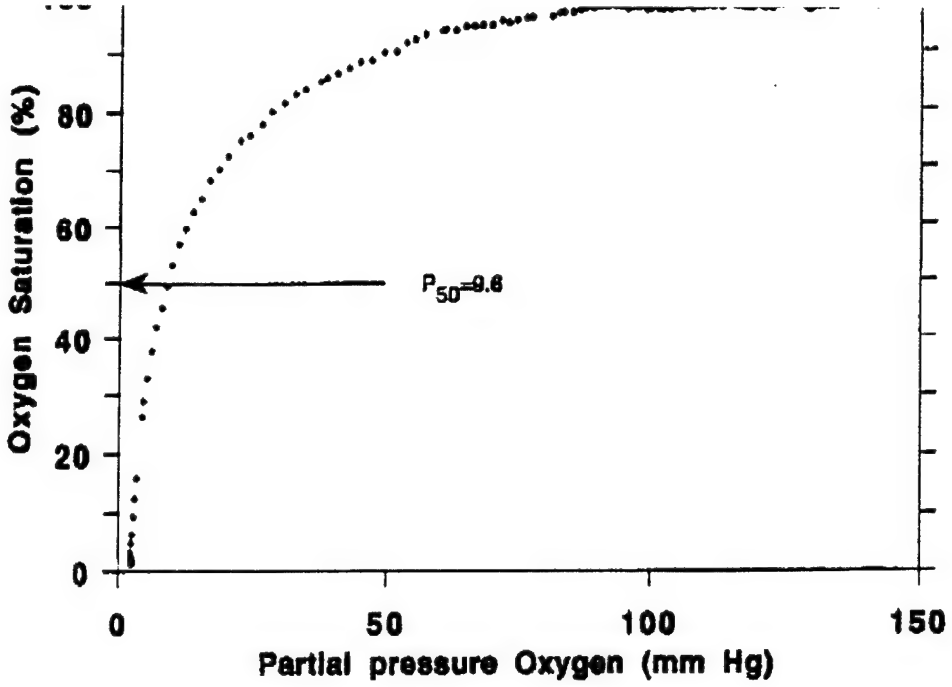

Oxygen Saturation (%)
80
60
40
20
0
P_{50}=9.6
0
50
100
150
Partial pressure Oxygen (mm Hg)

Fig. 16

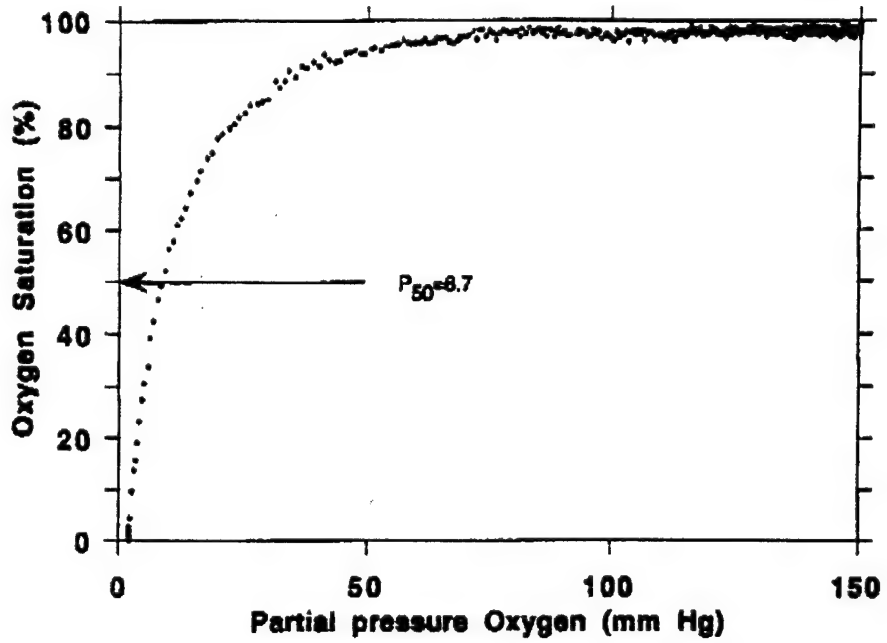

100
Oxygen Saturation (%)
80
60
40
20
0
P_{50}=6.7
0
50
100
150
Partial pressure Oxygen (mm Hg)

Fig. 17

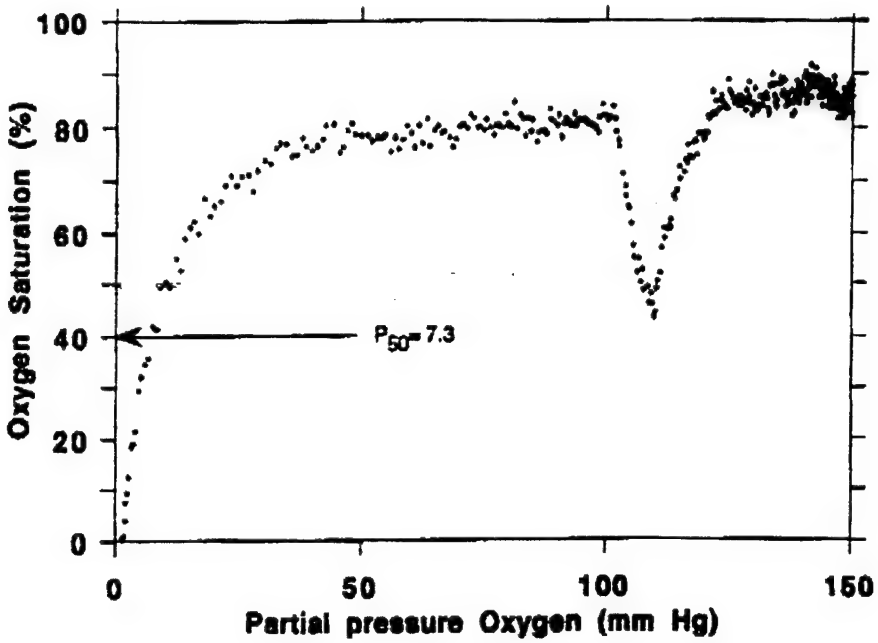
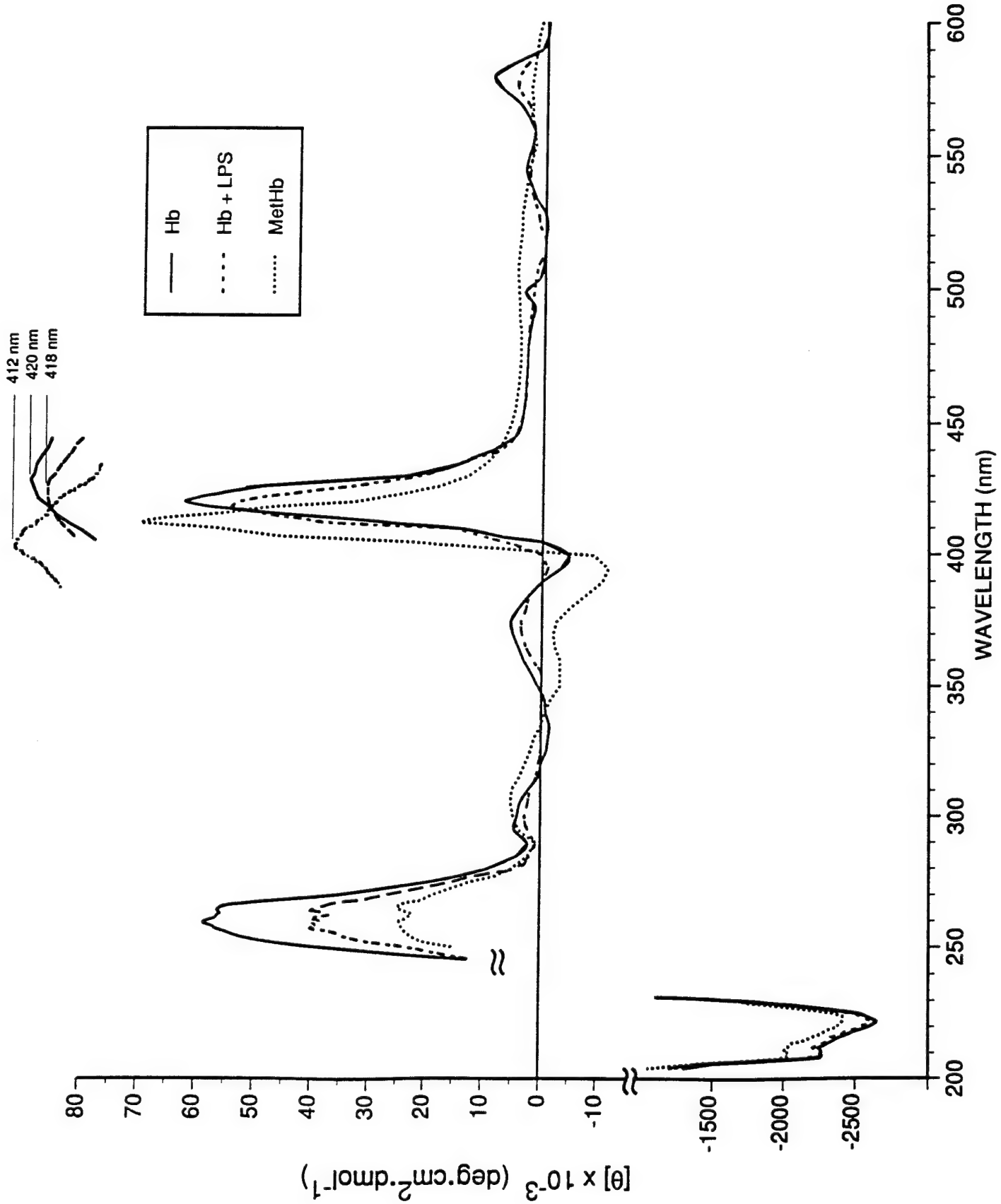

100
80
60
40
20
0
Oxygen Saturation (%)
P_{50}= 7.3
0
50
100
150
Partial pressure Oxygen (mm Hg)

Fig. 18

Fig. 19


Hb
Hb + LPS
MetHb
412 nm
420 nm
418 nm
WAVELENGTH (nm)
200
250
300
350
400
450
500
550
600
80
70
60
50
40
30
20
10
0
-10
-1500
-2000
-2500
[θ] x 10-3 (deg.cm2.dmol-1)

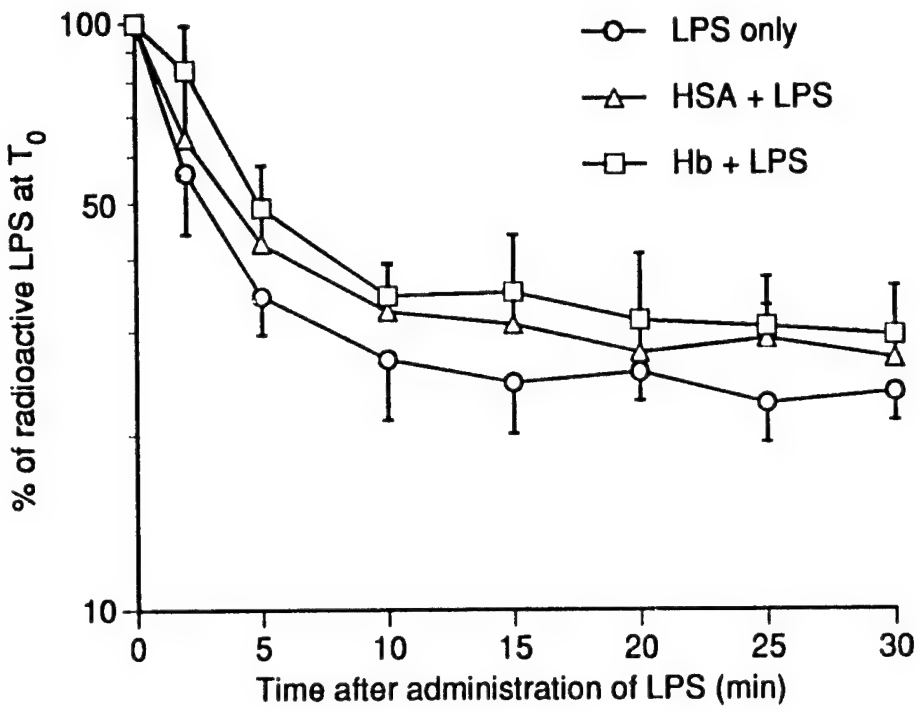

100
50
10
% of radioactive LPS at T_0
0
5
10
15
20
25
30
Time after administration of LPS (min)
LPS only
HSA + LPS
Hb + LPS

Fig. 20

TABLES

Table 1. Experiment: Affinity constants (K_d) were determined for the association of radiolabeled E. coli LPS with ααHb as measured by (1) a binding assay using immobilized Hb and (2) co-migration of LPS and Hb through 5% sucrose.

	K_d
BINDING TO MICROTITER PLATE	4.7×10^{-4} (g/L) (3.1×10^{-8} M)
REDISTRIBUTION AFTER SUCROSE CENTRIFUGATION	6.3×10^{-4} (g/L) (6.3×10^{-8} M)

Conclusion: LPS binds to Hb with moderately high affinity.

Table 2. Experiment: LPS was incubated with ααHb, and the mixtures subjected to polyacrylamide gel electrophoresis in the absence of SDS. Under these conditions, LPS-Hb complexes remained intact, and Hb migrated into the gel as a broad but distinct band. The percent of LPS entering this non-denaturing gel and co-migrating with ααHb was determined using radiolabeled LPS. In the absence of ααHb, all LPS remained in the stacking gel.

[a] Salmonella typhimurium ^{14}C-LPS [b] E. coli ^{125}I-LPS

	% LPS co-migrating with Hb
EXPERIMENT 1	43 [a]
EXPERIMENT 2	45 [a]
EXPERIMENT 3	23 [b]

Conclusion: LPS-Hb complexes electrophorese similarly to Hb alone in a non-denaturing gel. LPS in these complexes has been disaggregated.

Table 3. Experiment: A variety of LPSs were assayed for LAL activity in the absence and presence of ααHb. LAL reactivity of each LPS alone was designated as 1. Fold enhancement of each LPS by Hb is reported.

	Fold Enhancement
Biologically active LPSs	
E. coli 026:B6, crude	58
E. coli 026:B6, Na salt	62
E. coli 026:B6, Ca salt	65
E. coli 026:B6, triethylamine salt	58
E. coli Re F515	9
S. abortus equi	8
Non-toxic LPSs	
Rhodobacter spheroides	1
Rhodobacter capsulatus	1
Rhodopseudomonas viridis	1

Conclusion: Hb enhancement of LAL activity was a generalized phenomenon for a variety of biologically-active LPSs. Non-toxic LPSs, which have minimal endotoxic properties in other biological assays, were unaffected by Hb.

Table 4. Experiment: The effect of Hb on the sensitivity of LPS detection by the LAL test was investigated. LPSs were assayed by the LAL test in the absence and presence of ααHb or HbA_0, and the lowest detectable LPS concentrations determined. For both LPSs tested, the fold-increase in sensitivity in the presence of Hb was calculated from the ratios of the lowest detectable LPS concentrations (+Hb/-Hb).

	Fold increase in sensitivity for LPS
E. coli LPS + ααHb	15
E. coli LPS + HbA_0	17
P. mirabilis LPS + ααHb	11
P. mirabilis LPS + HbA_0	9

Conclusion: The limit of LPS detection was lowered >10-fold in the presence of Hb.

Table 5. Experiment: Oxygen affinity measurements were obtained for crosslinked (ααHb) and native (HbA_0) hemoglobins alone or in the presence of LPS after 2-hr incubation at 37°C. Measurements were obtained utilizing both smooth and rough LPSs: [a] P. mirabilis 03 (smooth) LPS; [b] S. minnesota Re 595 (rough) LPS; [c] E. coli 026 (smooth) LPS; [d] S. minnesota OH37 (rough) LPS.

	P_{50}
ααHb alone	26.6
ααHb + LPS[a]	25.1
ααHb + LPS[b]	25.6
HbA_0 alone	9.6
HbA_0 + LPS[c]	8.7
HbA_0 + LPS[d]	7.3

Conclusion: In the presence of LPS, each Hb demonstrated a trend toward higher oxygen affinity.

Table 6. Experiment: Summary of the effect of S. minnesota OH37 LPS on ααHb secondary structure.

	$[\theta]_{222} \times 10^{-3}$	Calculated % α-helix
ααHb	-2646	53
ααHb/LPS	-2616	52
MetHb	-2413	48

Conclusion: There was no effect of LPS on the α-helical content of ααHb. (See Fig. 19).

MEETINGS ATTENDED

International Endotoxin Society(Third Conference)
Helsinki, Finland August, 1994

Blood Substitutes & Related Products. Advances in Development, Trial Design & Clinical Application
Washington, DC September, 1994

Current Issues in Blood Substitute Research and Development
San Diego, CA March, 1995

Outline of Presentation

Evidence for complex formation between human hemoglobin and bacterial endotoxin
- Saturable binding of endotoxin to immobilized hemoglobin
- Photoaffinity labeling of hemoglobin with a endotoxin photoaffinity probe
- Co-migration of endotoxin and hemoglobin by centrifugation and electrophoresis

Effect of complex formation on the biological activity of bacterial endotoxin
- Enhancement of activation of Limulus amebocyte lysate
- Enhancement of production of tissue factor by human peripheral blood mononuclear cells
- Enhancement of production of tissue factor by human vascular endothelial cells

(Blood Substitutes & Related Products)

The Effects of Bacterial Endotoxins on Human Hemoglobin

Jack Levin, M.D.

SUMMARY

Previous investigations have demonstrated that hemoglobin (Hb) is a binding protein for bacterial endotoxin (lipopolysaccharide, LPS), and that the structure and biological activity of LPS are altered in the presence of Hb. In the present study, the influence of LPS on the structure of native human HbA_0 and covalently crosslinked Hb ($\alpha\alpha$Hb) has been studied by spectral analysis of Hb in the Soret and visible regions. Incubation of oxyHb with each of several LPSs resulted in a decrease in the intensity of the major Soret band at 414 nm with a shift in the maximum peak to 410 nm, decreases in the intensities of the major visible region peaks at 541 nm and 577 nm, and the appearance of increased absorbance in the visible region in the range of 630 nm. The resultant spectra are characteristic of methemoglobin formation. These spectral changes were time-dependent and LPS-concentration dependent. Production of methemoglobin was prominent with chemically-modified, partially deacetylated rough LPS, and was observed to a lesser extent both with native, complete rough and native smooth LPSs. The influence of LPS on the absorption spectra of methemoglobin also was directly tested. The conversion of methemoglobin to hemichrome in the presence of LPS was demonstrated and was shown to be reversible. In conclusion, analyses of Hb absorption spectra reveal the potential of LPS to produce a facilitated degradation of both $\alpha\alpha$-crosslinked human Hb and native human HbA_0.

(Blood Substitutes & Related Products)

A33 LPS INFUSION IN THE CYNOMOLGUS MONKEY: EFFECTS OF TNF BLOCKADE ON LEUKOCYTE DYNAMICS. G. Jesmok*, M. Fournel and R. Gundel
Miles Inc. West Haven Ct USA

Low dose LPS infusion (4-5 ng/kg) in humans and chimpanzees results in a putative systemic inflammatory response which may have relevance to the development of septic shock. LPS at these low doses results in moderate, readily reversible hemodynamic changes (increased HR and Temp), an elevation of inflammatory cytokines (IL-6, IL-8) and a rapid leukocytosis reaching 2-3 fold by 5 hrs. In the chimpanzee, TNF blockade with a neutralizing antibody diminished the production of IL-6 and IL-8, but had no effect on the leukocytosis. Since the leukocytosis induced by low dose LPS was not associated with any pathologic hemodynamic alteration or residual morbidity, we speculated that a higher dose of LPS may be needed to induce physiologic changes which more closely resemble septic shock. We therefore infused LPS at a higher dose (1 ug/kg over 15 minutes) and monitored hemodynamics and leukocyte dynamics over 5 hrs in the presence and absence of TNF blockade with MAb (BAY X 1351, 7.5 mg/kg). While low dose LPS (10 ng/kg) was associated with no hemodynamic changes or morbidity and a profound leukocytosis similar to the chimpanzee, higher doses of LPS were associated with hemodynamic instability (hypotension), morbidity, and leukopenia (2-4 hrs). These pathogenic changes were abrogated with TNF MAb treatment. These results suggest that low dose LPS infusion in primates may not be an appropriate model for the development of septic shock complications since it is the hyperadhesive state of the vascular endothelium and/or leukocyte as reflected by the prolonged leukopenia (induced by higher doses of LPS) which appears to be more closely associated with hemodynamic instability and morbidity. Furthermore, and of therapeutic interest, it is this pathologic systemic inflammatory response which is modified by TNF blockade.

A34 HEMOGLOBIN: A NEWLY RECOGNIZED LIPOPOLYSACCHARIDE BINDING PROTEIN

W. Kaca, R.I. Roth* and J. Levin

University of California School of Medicine, San Francisco, CA, and the Institute of Microbiology and Immunology, University of Lodz, Lodz, Poland

Cell-free hemoglobin (Hb) is a purified preparation of human hemoglobin that is being developed as a resuscitation fluid. In vivo administration of hemoglobin has resulted in significant toxicity, due in part to contamination with bacterial endotoxin (lipopolysaccharide, LPS). To better understand this toxicity, we have studied the interaction between Hb and LPS. Mixtures of each of three different Hb preparations (crosslinked $\alpha\alpha$Hb, crosslinked carbonmonoxy $\alpha\alpha$HbCO, and non-crosslinked (native) HbA_0) and LPS (E. coli O26:B6 or P. mirabilis S1959) were examined by several independent methods for evidence of Hb-LPS complex formation. Binding assays in microtiter plates demonstrated saturable binding of LPS to immobilized Hb, with a kD of 3.1×10^{-8}M. Binding of LPS to Hb also was demonstrated with a radiolabeled LPS photoaffinity probe. Ultrafiltration of Hb-LPS mixtures by 300 kDa and 100 kDa cut-off membranes showed that the majority of LPS in these mixtures (87-97% and 64-72%, respectively) was detected in the filtrates, in contrast to the lack of filterability of LPS in the absence of Hb. Density centrifugation showed that LPS co-migrated with each of the three Hbs, whereas unbound LPS had a distinctly greater sedimentation velocity than Hb or Hb-LPS complexes. Non-denaturing polyacrylamide gel electrotrophoresis demonstrated that in the presence of Hb LPS migrated into the gel and co-electrophoresed with Hb, whereas LPS alone did not appreciably enter the gel. Finally, precipitation by ethanol of each of the three Hb preparations was increased in the presence of LPS compared to precipitation in the absence of LPS. In conclusion, our data provide several lines of evidence for Hb-LPS complex formation, and demonstrate that complex formation is accompanied by disaggregation of the LPS macromolecule.

A35 EFFECTS OF BACTERIAL ENDOTOXIN ON HUMAN CROSSLINKED AND NATIVE HEMOGLOBINS

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University of California School of Medicine, San Francisco, CA, University of California School of Medicine, San Diego, CA, and the Institute of Microbiology and Immunology, University of Lodz, Lodz, Poland.

Previous investigations have demonstrated that hemoglobin (Hb) is a binding protein for bacterial endotoxin (lipopolysaccharide, LPS), and that the structure and biological activity of LPS are altered in the presence of Hb. In the present study, the influence of LPS on the structure of native human HbA_0 and covalently crosslinked Hb ($\alpha\alpha$Hb) has been studied by spectral analysis of Hb in the Soret and visible regions. Incubation of oxyHb with each of several LPSs resulted in a decrease in the intensity of the major Soret band at 414 nm with a shift in the maximum peak to 410 nm; decreases in the intensities of the major visible region peaks at 541 nm and 577 nm; and the appearance of increased absorbance in the visible region in the range of 630 nm. The resultant spectra are characteristic of methemoglobin formation. These spectral changes were time-dependent, clearly appearing within 10 minutes, and LPS concentration dependent between 0.01 mg/ml and 1 mg/ml LPS. After one hr, greater than 50% of the starting $\alpha\alpha$Hb or HbA_0 had been converted to methemoglobin and hemichrome. Production of methemoglobin was most prominent with chemically-modified, partially deacylated rough LPS, and was observed to a lesser extent both with native, complete rough and native smooth LPSs. The influence of LPS on the absorption spectra of methemoglobin also was directly tested. The conversion of methemoglobin to hemichrome in the presence of LPS was demonstrated, and was shown to be reversible. In conclusion, analyses of Hb absorption spectra reveal the potential of LPS to produce a facilitated degradation of both $\alpha\alpha$-crosslinked human Hb and native human HbA_0 to methemoglobin and subsequently hemichrome.

A36 HUMAN HEMOGLOBIN INCREASES THE ABILITY OF BACTERIAL ENDOTOXIN TO ACTIVATE LIMULUS AMEBOCYTE LYSATE AND ENDOTHELIAL CELLS

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Preparations of human hemoglobin (Hb) are being developed for use as an oxygen-transporting resuscitation fluid. Previous studies demonstrated that Hb and bacterial endotoxin (lipopolysaccharide, LPS) formed stable complexes, in which macromolecular LPS had become disaggregated. To examine the effect of complex formation on LPS biological activity, we investigated the ability of Hb to alter activation of the coagulation cascade of Limulus amebocyte lysate (LAL) by LPS or the LPS-stimulated formation of tissue factor from endothelial cells. Both native Hb and derivatized (covalently crosslinked) Hb produced prominent, protein concentration dependent enhancement of LAL activation and endothelial cell tissue factor production by Proteus mirabilis LPS. There were no substantial differences between the enhancement effect of Hb on Proteus mirabilis smooth and rough LPSs, suggesting that the lipid A component of LPS, but not the O-chain saccharide component, played an important role in the enhancement process. Rough (Re) Salmonella minnesota 595 LPS also demonstrated enhanced activation of LAL and stimulation of endothelial cell tissue factor in the presence of Hb. In contrast, lipid A and singly dephosphorylated or partially deacylated Re595 LPS showed little or no enhancement of LAL activation by Hb, and partially deacylated lipid A showed no enhancement of endothelial cell tissue factor by Hb. These results indicated that the Kdo moieties, as well as the phosphate residues and fatty acyl moieties of lipid A, were involved in enhancement of biological activity by Hb. Comparison of Hb with other endotoxin binding proteins for ability to cause enhancement of LPS biological activity demonstrated more prominent enhancement by lipopolysaccharide binding protein (LBP) than that observed with Hb, lesser enhancement with albumin, and no enhancement effect at all by IgG or transferrin.

A37 SIGNIFICANCE OF SOLUBLE ADHESION MOLECULES (sICAM-1, sELAM-1, sVCAM-1) IN SEPSIS AND SEPTIC MULTIPLE ORGAN FAILURE (MOF)

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Activated endothelial cells release sICAM-1, sELAM-1, and sVCAM-1. sICAM-1, sELAM-1, sVCAM-1, TNF-α, IL-6, and IL-8 in severe injuried patients were measured by ELISA. Endotoxin was measured by an endotoxin-specific Endospecy test.

The sICAM-1 and sVCAM-1 levels were significantly higher in the septic MOF and sepsis groups than in the non-septic MOF group.

The sELAM-1 level was slightly higher in the septic MOF group than in the sepsis without MOF group and non-septic MOF group.

The increases of soluble adhesion molecules were not in agreement with changes of plasma endotoxin level. It was possible that the release of soluble adhesive molecules were not stimulated by plasma endotoxin, but endotoxin in the local infectious region. However, levels of soluble adhesion molecules were correlated with the levels of plasma TNF-α, IL-6, and IL-8. These cytokines were suggested to be involved in the release of these soluble adhesion molecules.

The sICAM-1 and sVCAM-1 levels in septic patients closely reflected the severity of the pathophysiological condition. It was suggested that these soluble adhesive molecules were released from endothelial cells activated by stimuli due to infection..

A38 SIMULTANEOUS ASSESSMENT OF KUPFFER CELL PHAGOCYTIC AND SECRETORY FUNCTION IN OBSTRUCTIVE JAUNDICE

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Introduction Impaired Kupffer cell phagocytic function contributes to the pathophysiology of septic complications in obstructive jaundice. Cytokines (TNF and IL6) produced by the Kupffer cell may also be important mediators of the septic response. This study investigates the relationship between Kupffer cell clearance capacity (KCCC) and secretory function in experimental obstructive jaundice.

Methodology Male Wistar rats (n=16, weight 250-300g) underwent bile duct ligation (B) or sham operation (S). After 21 days Kupffer cell function was assessed using *in situ* hepatic perfusion. Livers were perfused (30ml/min) for 10 minutes with fluorescein labelled endotoxin (1.6 μg/ml) and then for a further fifty minutes with endotoxin free perfusate. KCCC was determined from the fluorescence ratio of influent and effluent perfusate during the initial ten minute period. Secretory function was evaluated by assaying effluent perfusate for TNF (ELISA) and IL6 (bioassay) sampled at 20 and 60 minutes.

Results Data expressed as mean (standard error mean)

	KCCC	20 min		60 min	
	%	TNF pg/ml	IL6 pg/ml	TNF pg/ml	IL6 pg/ml
S	41.4(2.3)	8.1(22.9)	0	112(18)	0
B	22.2(3.4)*	12.3(12.3)	4.8(4.8)	1140(133)*	102(31.4)*

* $p<0.05$ vs S (Student's *t* test)

Conclusions Following three weeks of obstructive jaundice KCCC is impaired but secretion of pro-inflammatory cytokines is enhanced. These simultaneous, but paradoxical Kupffer cell responses may be important contributors to the pathophysiology of septic complications in obstructive jaundice.

PERSONNEL

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APPENDIX

ANNUAL REPORT
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JACK LEVIN, M.D., Principal Investigator

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Distribution of Bacterial Endotoxin in Human and Rabbit Blood and Effects of Stroma-Free Hemoglobin

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Bacterial endotoxin (lipopolysaccharide [LPS]) is known to interact with numerous components of blood, including erythrocytes, mononuclear cells, platelets, neutrophils, lipoproteins, and plasma proteins. The relative affinities of LPS for these elements, and the distribution of LPS between them, are unknown. Cross-linked stroma-free hemoglobin (SFH), a potential substitute for erythrocyte transfusion, produces in vivo toxicity in animals consistent with significant LPS contamination. Therefore, we studied the distribution of LPS in human and rabbit blood and examined whether the presence of SFH altered LPS distribution. In either the presence or absence of SFH, LPS was associated predominantly with high-density lipoproteins and apoproteins. There was lesser binding to low- and very-low-density lipoproteins. Examination of the apoprotein pool by column chromatography and density centrifugation demonstrated that LPS in this fraction was predominantly protein bound. Binding of LPS to SFH resulted in dissociation of a portion of the LPS into low-molecular-weight complexes. Cell-bound LPS was only 2 to 16% of the total and was unaffected by SFH. The distribution among blood cells demonstrated predominant binding to platelets in human blood but predominant binding to erythrocytes in rabbit blood. Cellular distribution was not significantly altered by SFH.

Bacterial endotoxin (lipopolysaccharide [LPS]) is the cell wall component of gram-negative bacteria responsible for initiation of fever, cardiovascular shock, and disseminated intravascular coagulation during septicemia. Endotoxin can enter the peripheral circulation at sites of wounds or the portal circulation by absorption and/or translocation from the gastrointestinal tract (30). When endotoxin is administered parenterally to animals, much of the injected LPS is initially found in the cell-free fraction of plasma, with special affinity for high-density lipoproteins (HDL) (12). Prominent binding to HDL has been shown in vitro (28, 46). LPS association with protein also has clearly been demonstrated (41, 50). Nevertheless, it has been known for several decades that LPS, when introduced into the blood of experimental animals, is rapidly cleared from the circulation (19, 23, 25).

Interactions between LPS and circulating blood cells are of great interest since many of the deleterious effects of LPS during septicemia are the result of mediators released from inflammatory cells. LPS causes the release of a wide range of cell-derived substances, including cytokines (e.g., tumor necrosis factor [1, 7, 26, 43], interleukin-1 [36, 48, 49], and interleukin-6 [36, 48, 49]), eicosanoids (22), and procoagulants (e.g., tissue factor [34]). However, animal studies have resulted in substantially discordant descriptions of the distribution of LPS among the various types of circulating blood cells. Injected LPS has been reported to associate preferentially with buffy coat cells (3), platelets (19), platelets, monocytes, and polymorphonuclear leukocytes (4), and monocytes and polymorphonuclear leukocytes (23) or to be uniformly distributed between all cellular elements (24). LPS interaction with erythrocytes has been clearly demonstrated in vitro (38–40), although most in vivo animal studies of LPS distribution in blood have failed to demonstrate binding to erythrocytes. The quantitative distribution of LPS in human blood has not been reported.

There is experimental evidence that plasma proteins may influence the binding of LPS to other elements in blood, possibly by disaggregation of LPS (46) and/or by enhancement of the ability of LPS to bind to HDL (42). Alteration of LPS by protein binding could potentially affect the clearance of LPS and/or the ability of LPS to stimulate the release of effector molecules from circulating blood cells (37, 47). Cross-linked stroma-free hemoglobin (SFH) (defined in Addendum in Proof) is an oxygen-carrying protein being developed as an erythrocyte substitute for which endotoxin binding and a resultant synergistic toxicity are major concerns (51, 52). When used as a resuscitation fluid after trauma, 100 g or more of SFH would potentially be infused into a patient. Endotoxin is likely to be present in the circulation during resuscitation after trauma because of skin and gut wounds and/or ischemia of the gastrointestinal tract. In addition, the infused SFH may have been contaminated by LPS during its production (10). Therefore, the effect of SFH on the distribution of LPS in blood is an unknown but important variable during resuscitation therapy and of potentially great clinical relevance. The current study was undertaken to quantify the distribution of LPS in human blood and to determine if the distribution was altered by the presence of SFH.

MATERIALS AND METHODS

Reagents and labware. Percoll (adjusted to $d = 1.07$ or $d = 1.09$ g/ml) and Ficoll (adjusted to $d = 1.07$ g/ml) were purchased from Pharmacia LKB (Alameda, Calif.), and Mono-poly resolving medium was from Flow Laboratories (ICN Biomedicals, Inc., Costa Mesa, Calif.). Citrated blood tubes and Falcon centrifuge tubes (sterile, 15 ml) were obtained from Becton Dickinson (Mountain View, Calif.), and sterile phosphate-buffered saline (PBS) was from GIBCO Laboratories (Grand Island, N.Y.).

LPSs. ^{14}C-LPS [*Salmonella typhimurium* PR122(Rc),

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1 μCi/mg] was purchased from List Biologicals, Inc. (Campbell, Calif.). ^{125}I-LPS (1 μCi/μg) was prepared from *Escherichia coli* O55:B5 LPS B as described previously (44). Gamma counting was performed in an automatic gamma counter (LKB Instruments, Inc., Gaithersburg, Md.), and ^{14}C scintillation counting was performed, after samples were diluted 10-fold in fluor (formula A-989; NEN Research Products, Boston, Mass.), in an analytic liquid scintillation system (Tracor Analytic, Elk Grove Village, Ill.). ^{14}C was detected at 1.72×10^6 counts per minute (cpm) of ^{14}C per μCi by this instrument.

SFH. Human cross-linked SFH, prepared as described previously (53), was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research, San Francisco, Calif. Cross-linking between the alpha chains was produced by derivatization with bis-(3,5-dibromosalicyl)fumarate. The SFH (7-g/dl stock solution) used in these experiments was at a final concentration of 1.2 g/dl and contained less than 10 pg of endotoxin per ml (referenced to *E. coli* O55:B5 LPS B) as determined by the *Limulus* amebocyte lysate test (21).

Animals. Two- to three-kilogram New Zealand White female rabbits were purchased from Western Oregon Rabbit Co. (Philomath, Oreg.). C57BL mice (25 g) were purchased from Simonsen Laboratories, Inc. (Gilroy, Calif.).

Radiolabeled plasma protein preparation. Mice were injected intraperitoneally with 90 μCi of [^{35}S]methionine (NEN Research Products). Cell-free mouse plasma was obtained by centrifugation of anticoagulant-treated blood samples (3,000 × *g* for 20 min) obtained 16 h after injection. The resulting plasma contained 8.6×10^5 cpm/ml. By utilizing this protocol, radioactivity has been shown previously to be associated with plasma proteins (9).

Leukocyte and platelet counts. Leukocyte and platelet counts were determined with blood cell counters (Coulter Electronics, Inc., Hialeah, Fla.). Leukocyte differentials in human blood samples were obtained with an H1 particle counter (Technicon Instrument Corporation, Tarrytown, N.Y.). Leukocyte differentials in rabbit blood smears were determined by 500-cell manual counts of Wright-Giemsa-stained blood smears.

Fractionation of whole blood. The following procedure for the separation of the various types of blood cells was developed experimentally to provide concomitant (i) maximum recovery of platelets, mononuclear cells, polymorphonuclear cells, and erythrocytes, (ii) maximum purity of each cell preparation, and (iii) minimal cell damage. Unless otherwise stated, procedures for fractionation of human and rabbit blood were identical.

Blood samples were obtained after the subjects had fasted overnight. Five-milliliter samples of citrated blood were incubated with 0.6 ml of SFH or NaCl at room temperature for 10 min. Final SFH concentrations were 1.2 g/dl. These blood samples were then incubated with 25 μl of radiolabeled LPS (approximately 10^5 cpm) for an additional 15 min at room temperature and centrifuged in an Accuspin centrifuge (Beckman Instruments, Inc., Irvine, Calif.), with an AH-4 swinging-bucket rotor, at 600 × g for 3 min, to obtain platelet-rich plasma and a cell pellet. The plasma was then centrifuged at 1,300 × *g* for 20 min to obtain platelets, and the platelet pellet was washed three times with 5 ml of PBS at 600 × *g* for 3 min. The cell pellet from the initial whole-blood centrifugation was resuspended in PBS to 8 ml, layered over 3 ml of Ficoll (d = 1.070 g/ml), and centrifuged at 400 × g for 40 min. Mononuclear cells (monocytes and lymphocytes) were present in a band at the interface between the PBS (top) and the Ficoll (bottom) and were collected and washed once with 5 ml of PBS (250 × *g*) for 10 min. The pellet beneath the Ficoll (polymorphonuclear leukocytes and erythrocytes) was fractionated by either of the following procedures, with comparable results for recovery and purity. The Ficoll pellet was resuspended in an equal volume of platelet-free plasma and then layered on 3 ml of Mono-poly resolving medium (d = 1.140 g/ml) and centrifuged at 400 × *g* for 40 min. Polymorphonuclear leukocytes were present at the interface, and erythrocytes were present in the pellet at the bottom of the tube. The leukocyte band was washed with 5 ml of PBS (250 × *g* for 10 min) and then recentrifuged on 2 ml of Mono-poly resolving medium to further remove erythrocytes. Because of the subsequent unavailability of Flow Mono-poly resolving medium, a Percoll separation of the Ficoll pellet was also established. Human Ficoll pellet cells (see above) were layered on 3 ml of Percoll (d = 1.090 g/ml) and centrifuged at 400 × *g* for 15 min. Rabbit Ficoll pellet cells were layered on 3 ml of Percoll (d = 1.070 g/ml) and centrifuged at 400 × *g* for 15 min. Polymorphonuclear leukocytes, present at the plasma-Percoll interface, and erythrocytes in the pellet were washed once with 8 ml of PBS (250 × *g* for 10 min).

Erythrocyte-containing fractions exhibited quenching of both ^{125}I and ^{14}C cpm. Therefore, 0.1-ml aliquots of erythrocyte fractions were diluted 10-fold in water (final volume, 1 ml), and 1.0 ml of Solvable (NEN Research Products) was added. These mixtures were incubated at 60°C for 1 h, and then 0.3 ml of 25% H_2O_2 was added. After 30 min of additional incubation at room temperature, samples were pale yellow in color and could be analyzed for radioactivity. Recovery of a spiked radioisotope in preliminary experiments demonstrated >98% detection of the previously added radioactivity.

All cell preparations were cytocentrifuged (Shandon Southern Instrument Co., Sewickley, Pa.), and 200 to 500 cell differentials were performed to determine purity.

Separation of lipoproteins from cell-free plasma. Separation of platelets from human or rabbit plasma, as described above, yielded cell-free plasma which contained less than 0.1% of starting platelets and undetectable numbers of leukocytes or erythrocytes (determined by Coulter counter analysis and examination of Wright-Giemsa-stained smears). The cell-free plasma was subjected to sequential ultracentrifugation (35) at 4°C at plasma density (d = 1.006 g/ml), at a density of 1.063 g/ml (with KBr), and at a density of 1.21 g/ml (with KBr) for isolation of very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and HDL, respectively, and for isolation of apoproteins (i.e., plasma proteins remaining after sequential removal of all lipoproteins; d > 1.21 g/ml).

Fractionation of plasma apoproteins. Cell-free, lipoprotein-free proteins (d > 1.21 g/ml; see above) were dialyzed and concentrated in a concentration cell (Amicon Division, W. R. Grace & Co., Danvers, Mass.) with a 12-kDa-cutoff membrane for gel filtration chromatography on Sephadex G-150 (100 by 2.6 cm). Chromatography was performed in PBS at room temperature.

Fractionation of endotoxin complexes. Unbound LPS was separated from protein-LPS complexes (and free protein) by centrifugation through a cushion of 20% sucrose. A 1- to 3-ml sample was layered over 4 ml of 20% sucrose and centrifuged at 25,000 × *g* for 1 h at 20°C in a Sorvall RC-5 centrifuge (Du Pont Instruments, Wilmington, Del.). Unbound LPS predominantly sedimented to the bottom of the

TABLE 1. Distribution of endotoxin in whole blood[a]

WB and endotoxin type	Endotoxin distribution						n^b
	% Total cpm		% cpm in cellular compartment				
	Plasma	Cells	PLTS	MNC	PMN	RBC	
Human WB							
^{125}I-*E. coli* O55:B5	98 ± 1	2 ± 1	77 ± 15	10 ± 10	3 ± 2	10 ± 10	9
^{14}C-*S. typhimurium*	96 ± 5	4 ± 5	55 ± 24	23 ± 5	14 ± 25	8 ± 6	5
^{14}C-*S. typhimurium* + SFH (1.2 g/dl)	96 ± 4	4 ± 4	37 ± 20	36 ± 14	15 ± 23	12 ± 10	9
Rabbit WB							
^{14}C-*S. typhimurium*	84 ± 16	16 ± 15	3 ± 3	15 ± 14	4 ± 3	78 ± 15	14
SFH (1.2 g/dl)	90 ± 10	10 ± 11	5 ± 5	23 ± 31	4 ± 4	68 ± 29	10
% Recovery of cells			49 ± 21	14 ± 10	62 ± 30	>99	

[a] Radiolabeled endotoxin was added to whole blood (WB), and the blood was then fractionated into cell-free plasma, platelets (PLTS), mononuclear cells (MNC), polymorphonuclear leukocytes (PMN), and erythrocytes (RBC). Endotoxin distributions are expressed as means ± SD. Recovery of added cpm was 98% (mean value).

[b] *n*, number of independent experiments.

tube under these conditions, whereas protein-LPS complexes remained above the sucrose layer.

RESULTS

Distribution of endotoxin in human blood. The in vitro distribution of endotoxin in human blood was studied, in the presence or absence of SFH (defined in Addendum in Proof), by using radiolabeled *E. coli* O55:B5 LPS B or *S. typhimurium* LPS. In the absence of SFH, almost all of either of the radiolabeled endotoxins (96 to 98%) was associated with cell-free plasma (Table 1). Because of the predominance of plasma-associated counts, it was necessary to develop a procedure for isolating blood cells free of plasma to accurately determine cell-bound LPS. The procedure for cell isolation described in Materials and Methods was tested with whole blood to which ^{35}S-labeled mouse plasma proteins (0.08 mCi; see Materials and Methods) had been added. Blood cells were isolated (see Materials and Methods) and ^{35}S cpm were measured to determine the percentage of plasma contamination in each cell pool. Platelets were shown to be associated with only 0.015% of the total plasma cpm initially added; mononuclear cells were associated with 0.008%; polymorphonuclear leukocytes were associated with 0.008%; and erythrocytes were associated with 0.001%. This level of plasma contamination was equivalent to only 1 to 3% of the cpm detected in the samples of isolated blood cells, thus ensuring that the endotoxin detected with cells was actually cell bound and did not represent contamination by plasma endotoxin. This extremely low level of plasma contamination of blood cell samples was accomplished by the extensive washing steps described in Materials and Methods; consequently, recoveries of cells were diminished because of the multiple wash steps. Recoveries of cells, from a total of 47 individual experiments (23 human and 24 rabbit), were as follows: platelets, 49% ± 21%; mononuclear cells, 14% ± 10%; polymorphonuclear leukocytes, 62% ± 30%; and erythrocytes, >99%.

In human blood, with both LPSs, 2 to 4% of the counts were cell associated (Table 1). In all experiments (n = 23), the majority of the cell-associated endotoxin cpm was found in the platelet pool. Distribution among the remaining cell types was quite variable between experiments. Blood from five normal human volunteers was utilized, and no reproducible differences in endotoxin distribution among the types of blood cells were detected between individuals. In addition, LPS distributions were the same in heparinized and citrate-treated blood samples from the same individual. Because of the predominance of platelet-associated cpm blood cell differentials were determined to ensure that cpm in the leukocyte and erythrocyte samples did not represent platelet contamination. Mononuclear cell preparations contained 76% ± 15% lymphocytes and monocytes, 1% ± 1% polymorphonuclear leukocytes, 17% ± 13% erythrocytes, and 6% ± 13% platelets (means ± standard deviations [SD], 15 experiments). Polymorphonuclear leukocyte preparations contained 65% ± 17% polymorphonuclear leukocytes, 34% ± 19% erythrocytes, 2% ± 7% platelets, and 0.1% ± 0.2% mononuclear cells (means ± SD, 15 experiments). The erythrocyte preparations contained greater than 99% erythrocytes, with less than 0.01% contamination with platelets. The low frequencies of platelets in the mononuclear leukocyte, polymorphonuclear leukocyte, and erythrocyte samples thus ensured that platelet-LPS contamination did not contribute to the presence of endotoxin in the preparations of the other types of circulating blood cells. Platelet-associated cpm contributed only 0.1% of the cpm in mononuclear cell preparations and 0.05% of the cpm in polymorphonuclear leukocyte preparations. Platelet preparations contained 99% ± 1% platelets (mean ± SD, 14 independent experiments).

The distribution of *S. typhimurium* endotoxin in human blood also was investigated in the presence of SFH. Almost all cpm (96%) were detected in cell-free plasma (Table 1), similar to the distribution observed in the absence of SFH. The distribution of endotoxin among the various types of blood cells (Table 1) demonstrated prominent binding to both platelets and mononuclear cells. In six of the nine experiments, most of the cell-associated cpm were again detected in the platelet pool. SFH appeared to produce an increase in the fraction of cell-bound LPS associated with mononuclear cells; however, because of the considerable variation of cellular distributions of endotoxin, differences between distributions in the presence and absence of SFH were not statistically significant.

Distribution of endotoxin in rabbit blood. The distribution of ^{14}C-labeled *S. typhimurium* endotoxin in rabbit blood, in the presence and absence of SFH, was determined in vitro. Almost all cpm were associated with the cell-free plasma (Table 1), similar to the distribution observed in human blood, although the total cell-associated endotoxin cpm were

TABLE 2. Endotoxin distribution in cell-free plasma[a]

Plasma and endotoxin type	Endotoxin distribution (% total cpm)				n^b
	VLDL	LDL	HDL	Apoproteins (lipoprotein-free pool)	
Human plasma					
S. typhimurium	2 ± 1	7 ± 1	68 ± 9	23 ± 7	4
S. typhimurium + SFH (1.2 g/dl)	2 ± 1	9 ± 7	67 ± 8	22 ± 3	4
Rabbit plasma					
S. typhimurium	3 ± 2	11 ± 11	50 ± 25	36 ± 3	4
S. typhimurium + SFH (1.2 g/dl)	2 ± 2	4 ± 4	53 ± 14	41 ± 14	4

[a] Radiolabeled endotoxin was added to whole blood in the presence or absence of SFH. Cell-free plasma was then prepared and fractionated by sequential ultracentrifugation steps into VLDL (d < 1.006 g/ml), LDL (d = 1.006 to 1.063 g/ml), HDL (d = 1.063 to 1.21 g/ml), and apoproteins (d > 1.21 g/ml; plasma proteins remaining after sequential removal of lipoproteins). Recovery of added cpm from the cell-free plasma was 85% (mean value). Endotoxin distributions are expressed as means ± SD.

[b] n, number of independent experiments.

in general greater in rabbit blood than in human blood. Sixteen percent of the total cpm was cell associated in the absence of hemoglobin (n = 14), and 10% was cell associated in the presence of hemoglobin (n = 10). However, this difference was not significant. The distribution among cells demonstrated that 78% of the endotoxin cpm in the absence of SFH (n = 14) and 68% of the endotoxin cpm in the presence of SFH (n = 10) were associated with the erythrocytes, a distinct difference from the predominance of platelet-associated endotoxin among the cells in human blood. The few erythrocytes that contaminated mononuclear cell preparations (17% of cells) and polymorphonuclear leukocyte preparations (34% of cells) contributed only 0.01 and 0.03% of the cpm in these cell preparations, respectively. Cell-associated endotoxin distributions in the presence and absence of SFH were not significantly different.

Distribution of endotoxin among the components of cell-free plasma. Cell-free plasma was fractionated by sequential ultracentrifugation steps, as described in Materials and Methods, into VLDL, LDL, HDL, and apoproteins (d > 1.21 g/ml). The distributions of ^{14}C-labeled *S. typhimurium* endotoxin among these components in both human and rabbit plasma samples and in the presence and absence of SFH are shown in Table 2. In both species, the relative magnitude of the distributions of endotoxin was HDL > apoproteins > LDL > VLDL.

Apoprotein fractions (d > 1.21 g/ml) from rabbit plasma samples containing ^{14}C-labeled *S. typhimurium* endotoxin were then fractionated by G-150 gel permeation chromatography. In the absence of SFH, all radioactivity eluted in the void volume (molecular mass, >200 kDa; Fig. 1). This pattern was detected in each of five independent samples chromatographed. Considerable A_{280} also was present in the void volume, and numerous protein bands were detected in this material by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (data not shown). In four apoprotein samples containing SFH, the majority of the cpm similarly was present in the void volume, although small amounts of radioactivity (5 to 24%) also were detected in the included volume (Fig. 2). SFH, as measured by A_{570}, was detected primarily in the included volume, although a small absorbance peak also was detected in the void volume. The retained peak of ^{14}C-endotoxin coeluted with the peak of SFH (Fig. 2). In contrast, ^{14}C-endotoxin alone eluted exclusively in the void volume (four experiments, data not shown). Thus, the endotoxin, which eluted in the included volume derived from samples containing SFH, was partially disaggregated.

Since the majority of cpm in the fraction of plasma with a density greater than 1.21 g/ml (i.e., which contained apoproteins) eluted in the void volume of Sephadex G-150, as did endotoxin alone, it was unclear whether endotoxin in this plasma fraction was protein bound. To distinguish unbound from bound endotoxin, sedimentation through sucrose was performed. Preliminary experiments demonstrated that unbound endotoxin sedimented through 20% sucrose (Table 3), under the conditions described in Materials and Methods, whereas plasma proteins or free SFH was predominantly less dense than the sucrose solution and, after centrifugation, remained in the aqueous layer above the sucrose cushion. Void volume fractions, from two samples to which SFH had not been added and two which contained SFH, were centrifuged over 20% sucrose. Endotoxin cpm in each of the four Sephadex G-150 void volume fractions remained predominantly in the layer above the sucrose, indicating that the endotoxin was comigrating with the proteins both in the presence and absence of SFH (Table 3). Endotoxin cpm from the included volume peak of a sample containing SFH similarly demonstrated that the endotoxin was primarily comigrating with protein in the layer above the sucrose (presumably SFH; compare Fig. 1 and 2).

To confirm the observation that endotoxin in the fraction of plasma with a density greater than 1.21 g/ml was protein bound, 11 additional apoprotein samples (5 without SFH and 6 with SFH) containing ^{14}C-LPS were generated. These fractions were then subjected to sedimentation through 20% sucrose without prior chromatography. Each of these samples demonstrated that the majority of endotoxin cpm was present in the aqueous phase above the sucrose cushion (Table 3). Most of the plasma proteins, as estimated by A_{280}, also were present in this top zone. These observations were consistent with a major decrease in density of endotoxin in the presence of plasma proteins, most likely due to a disaggregation of high-molecular-mass endotoxin macromolecules (typically greater than 1,000 kDa in the absence of protein) secondary to protein binding.

Since endotoxin was detected in the included volume of Sephadex G-150 chromatography in the presence of SFH (Fig. 2), it seemed likely that endotoxin had formed a specific complex with SFH. Therefore, we examined whether endo-

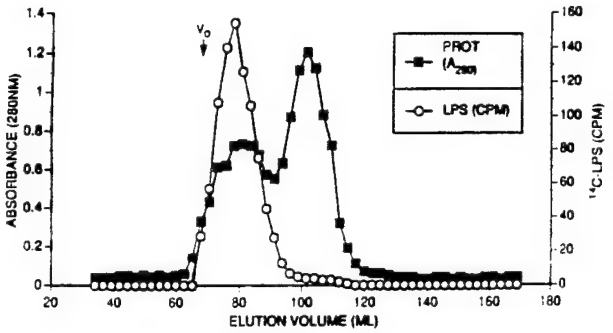

FIG. 1. Gel permeation chromatography of plasma apoproteins in absence of SFH. Plasma apoproteins (fraction with d of >1.21 g/ml from sequential density centrifugations of cell-free rabbit plasma) were chromatographed in the absence of SFH on Sephadex G-150. Proteins were monitored at A_{280} (closed squares), and endotoxin was monitored by determination of ^{14}C cpm (open circles). The void volume (V_0) is indicated.

toxin and SFH could form a stable complex in the absence of any other blood components. ^{14}C-labeled *S. typhimurium* LPS (6.8×10^4 cpm) was incubated with 0.5 ml of SFH (1.2 g/dl) at room temperature for 15 min. The mixture was then layered above 20% sucrose and centrifuged as described in Materials and Methods. Ninety-eight percent of the endotoxin cpm was detected in the SFH layer above the sucrose, 1.9% of the cpm was in the top three-fourths of the sucrose layer, and only 0.1% of the cpm was at the bottom of the sucrose layer. In contrast, a control tube of endotoxin in PBS demonstrated 97% of the endotoxin in the bottom fraction.

DISCUSSION

The relative affinities of LPS for specific cellular, lipoprotein, and apoprotein components of whole blood have not been described previously. Therefore, we performed in vitro studies of the distribution of endotoxin in samples of whole blood by using two purified, commonly studied enteric LPSs. In both human and rabbit blood, endotoxin associated primarily with the noncellular components of blood. Binding to HDL was greatest, followed by binding to apoproteins. Appreciable, although lesser, amounts of binding to LDL

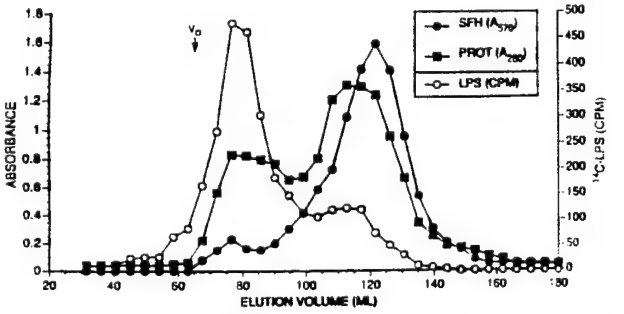

FIG. 2. Gel permeation chromatography of plasma apoproteins in the presence of SFH. Plasma apoproteins (fraction with d of >1.21 g/ml from sequential density centrifugations of cell-free rabbit plasma) were chromatographed in the presence of SFH (1.2 g/dl) on Sephadex G-150. Proteins were monitored at A_{280} (closed squares), SFH was monitored at A_{570} (closed circles), and endotoxin was monitored by determination of ^{14}C cpm (open circles). The void volume (V_0) is indicated.

TABLE 3. Sucrose sedimentation of apoproteins[a]

Fraction	Endotoxin distribution (% total cpm)		
	Top	Middle	Bottom
LPS alone (n = 4)	10 ± 1	4 ± 1	86 ± 13
G-150 V_0 fraction (apoproteins; n = 2)	77	12	11
G-150 V_0 fraction (apoproteins + SFH; n = 2)	82	2	6
G-150 included volume (apoproteins + SFH; n = 1)	87	8	5
Apoproteins (n = 5)	77 ± 10	17 ± 5	6 ± 2
Apoproteins + SFH (n = 6)	83 ± 6	13 ± 2	4 ± 2

[a] Apoproteins (d > 1.21 g/ml; plasma proteins remaining after sequential removal of lipoproteins) containing ^{14}C-LPS were obtained by centrifugation of cell-free rabbit plasma to remove blood cells and lipoproteins. These apoproteins, apoprotein fractions partially purified by Sephadex G-150 chromatography, and ^{14}C-LPS alone were centrifuged through 4 ml of 20% sucrose. After centrifugation at 25,000 × g for 1 h, cpm were determined in the layer above the sucrose cushion (top), the upper 3 ml of sucrose (middle), and the bottom 1 ml of sucrose (bottom). A_{280}s, as an estimation of protein concentration, also were measured in these sucrose layers and were 64 ± 7 (top), 12 ± 2 (middle), and 24 ± 5 (bottom). Recovery of cpm layered over sucrose in these studies was 83% (mean value). Means ± SD (when more than two independent experiments were performed) are shown.

and VLDL were observed. In human blood, cell-associated endotoxin was detected primarily with platelets. However, the platelet-bound cpm constituted only 1 to 2% of the total endotoxin cpm distributed throughout the blood. In rabbit blood, the cell-associated endotoxin was primarily bound to erythrocytes; this constituted 10 to 16% of the total endotoxin cpm distributed throughout the blood.

The demonstration of binding of LPS to platelets in humans, and to erythrocytes in rabbits, is potentially of relevance to the development of disseminated intravascular coagulation during endotoxemia. Several biological consequences of the interaction of LPS with mammalian platelets have been described previously, including the LPS-induced aggregation of human platelets (33), activation and secretion of platelet factor 3 (20), and secretion of 5-hydroxytryptamine (6). The latter two processes were described in rabbits (human platelets were not studied). Therefore, it is interesting that the endotoxins tested in our study had affinity for human platelets but not for rabbit platelets. Human platelets are less responsive to endotoxin than are rabbit platelets (29), but the pyrogenic response of humans to endotoxin exceeds that of rabbits (17). It is possible that these differences reflect species-specific cell membrane characteristics. The membranes of human platelets and megakaryocytes previously have been shown to differ biochemically from murine platelets and megakaryocytes by the presence of Fc receptors (and their absence on murine platelets and megakaryocytes) (32). In contrast, the murine cells demonstrate type 1 complement receptors, while the human cells do not (32). Whereas LPS binding to human platelets is likely to result in significant physiological effects on hemostasis, the significance of LPS binding to rabbit erythrocytes is less clear. However, a glycoprotein receptor on erythrocyte membranes that binds LPS has been described, and its potential role in the etiology of immune hemolysis during sepsis has been discussed (40).

Previous in vivo studies have demonstrated that much of administered LPS associates with HDL (12, 28, 46). The binding of high-molecular-weight LPS particles to HDL involves the dissociation of LPS (45), a process involved in

LPS detoxification, and has been shown to result in altered electrophoretic behavior of HDL (12). The role of an apoprotein factor(s) in LPS binding to HDL has been demonstrated (27, 42). LPS-binding protein appears to be one such apoprotein that is a mediator of LPS dissociation and binding to HDL (41). We also have shown prominent binding of LPS to HDL, as well as demonstrable, although lesser, binding to the other classes of lipoproteins. However, the lipoprotein binding of exogenously administered, isolated LPS may not totally mimic the distribution of shed LPS, associated with bacterial outer member structures, as demonstrated previously for the LPS of *S. typhimurium* (28) and *Neisseria meningitidis* (2). Shed LPS also may demonstrate variable lipoprotein binding depending on the presence or absence of bacterial membrane proteins (13). Although numerically less impressive, the binding to VLDL in our study also may be biologically significant. It has been shown previously that binding of LPS to triglyceride-rich lipoproteins (VLDL and chylomicrons) diminishes the potency of LPS in activation of the coagulation system in *Limulus* lysate (8). Furthermore, VLDL and chylomicrons protect mice from LPS-induced mortality (18). Since hypertriglyceridemia is one of the earliest abnormalities observed in blood during sepsis, this interaction between LPS and the triglyceride-rich lipoproteins may serve as a defense against endotoxemia.

The presence of SFH at a concentration of 1.2 g/dl did not appreciably alter the distribution of endotoxin among the various blood cell types, lipoproteins, and the apoprotein pool in either human or rabbit blood. However, since we demonstrated that endotoxin was able to bind to SFH, it is possible that the distribution of endotoxin among specific plasma proteins of the apoprotein pool was significantly altered by the presence of hemoglobin. Furthermore, in contrast to the binding of LPS to another apoprotein, aggregated immunoglobulin G, after which the density of the complex was identical to that of LPS alone (16), we have demonstrated that binding of LPS to SFH involves disaggregation of LPS (unpublished observations). Since the distribution of endotoxin among specific plasma proteins in the presence and absence of SFH is not known, further fractionation experiments will be required to determine if endotoxin binds to SFH preferentially. Several mammalian endotoxin-binding proteins in plasma have been documented previously, including a rabbit acute-phase LPS-binding protein (41), lysozyme (31), complement (5, 14), immunoglobulin (16), and albumin (15). We are now able to add SFH to this list of endotoxin-binding proteins.

The capacity of SFH to act as an endotoxin-binding protein may prove significant for the potential use of SFH as a blood substitute. Endotoxin contamination of SFH during its production and purification has been recognized (11; personal observations), and even low concentrations of endotoxin in preparations of SFH may prove clinically significant when liters of SFH solutions are infused into patients, especially those who are already hypotensive. It also is likely that endotoxemia will be present in many patients with trauma and shock who would be receiving this blood product. Gram-negative bacteremia associated with infection is another clinical situation in which endotoxin-hemoglobin binding may be a significant phenomenon. Endotoxin-hemoglobin complexes also may form as a result of the hemolysis that often occurs during the process of disseminated intravascular coagulation associated with gram-negative sepsis.

Importantly, our data indicate that the presence of SFH does not alter the association of endotoxin with mononuclear cells, an interaction which appears to play a critical pathophysiologic role in the production of the sepsis syndrome. However, further investigation will be required to determine if endotoxin-hemoglobin complexes differ from endotoxin alone in their ability to stimulate production or release of mononuclear cell factors involved in the response of the host to sepsis. Additional study also will be required to determine if endotoxin clearance from either the circulation or internal organs is altered by complex formation with hemoglobin.

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ADDENDUM IN PROOF

After the submission of this paper, it was determined that the term SFH (stroma-free hemoglobin) requires further definition in order to avoid confusion with the use of this term in the older literature. In this report, SFH refers to cell-free, ultrafiltered human hemoglobin free of detectable erythrocyte stroma and chemically cross-linked in order to improve intravascular persistence and prevent renal toxicity.

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Production of modified crosslinked cell-free hemoglobin for human use: the role of quantitative determination of endotoxin contamination

R.I. ROTH, J. LEVIN, K.W. CHAPMAN, M. SCHMEIZL, AND F.R. RICKLES

In vivo toxicity remains a major barrier to the successful use of cell-free hemoglobin (Hb) as an oxygen carrier in humans. Bacterial endotoxin (lipopolysaccharide, LPS) is known to contribute to the in vivo toxicity of Hb preparations, and the prevention of LPS contamination is a critical aspect of the effort to create an efficacious Hb blood substitute. Limulus amebocyte lysate assays for endotoxin were performed on multiple Hb samples from 26 independent production runs for the preparation of human crosslinked cell-free hemoglobin (αα Hb). High levels of LPS contamination (1->100 ng/mL) of αα Hb solutions were detected in multiple samples during many of the initial production runs. It was observed that LPS contamination of αα Hb solutions could occur at any step during the production sequence. Substantial enhancement by αα Hb of the biologic effects of LPS was demonstrated by two independent assays for endotoxin (the Limulus amebocyte lysate test and a mononuclear cell procoagulant assay), whereas LPS biologic activity was only slightly increased by human serum albumin and substantially diminished by IgG. These results suggest that the prevention of LPS-related toxicities in vivo may be more important to the clinical use of Hb solutions than to the use of other intravenous protein products. Therefore, it was encouraging to note that, with the careful monitoring for LPS in the production facility and in multiple samples during cell-free Hb production, sources of LPS contamination were recognized and the appropriate sites were made endotoxin-free. Numerous subsequent production runs were performed without appreciable LPS contamination. Therefore, careful monitoring of LPS contamination allows for the production of Hb solutions that are sufficiently free of LPS for clinical use. **TRANSFUSION** 1993;33:919–924.

Abbreviations: αα Hb = human crosslinked cell-free hemoglobin; BRD/LAIR = Blood Research Division/Letterman Army Institute of Research; Hb = hemoglobin; HSA = human serum albumin; LAL = Limulus amebocyte lysate; LPS = lipopolysaccharide; MNC(s) = mononuclear cell(s); TF = tissue factor.

A MAJOR FOCUS OF RESEARCH and development in the field of substitutes for red cell transfusion is the preparation of cell-free hemoglobin (Hb). Hb preparations currently under investigation have been shown to possess adequate oxygen-carrying and -delivery characteristics,[1] and the tetramer form of the Hb molecule has been successfully stabilized, by chemical crosslinking of the peptide chains, to provide adequate intravascular retention.[2] A remaining central issue for the clinical use of modified (i.e., crosslinked) Hb (αα Hb) is toxicity.[3,4] The administration of Hb solutions has resulted in hypertension and bradycardia;[5,6] decreased renal blood flow and glomerular filtration rate;[6-8] histologic evidence of renal glomerular and tubular damage;[8] pulmonary, hepatic and cerebral thrombosis;[9,10] hepatocellular and renal epithelial cell damage;[11] prolongation of the partial thromboplastin time;[6] a decrease in the circulating levels of factor VIII;[12] and thrombocytopenia.[9,12] These toxicities appear to be caused by the effects of Hb itself,[13] red cell stromal phospholipids (when present),[14] and/or bacterial endotoxin contamination.[10,14,15]

The potential contamination of Hb preparations by bacterial endotoxin (lipopolysaccharide, LPS) is of

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particular concern because of the ubiquitous presence of this molecule in water and chemical reagents and on surfaces and because of the multiplicity of its deleterious biologic effects. In addition, the biologic effects of LPS are typi-cally observed at extremely low concentrations (pg/mL-ng/mL LPS). Despite a variety of known techniques for the removal of endotoxin from solutions,[16] prevention of LPS contamination remains the most effective and economical approach to minimizing this important aspect of Hb toxicity. Therefore, we undertook efforts to produce αα Hb that was devoid of physiologically significant concentrations of LPS and potentially suitable for clinical trials. We performed assays to determine the concentrations of endotoxin in a large number of Hb and αα Hb solutions, as well as in a variety of wash solutions used in the Hb production facility at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR, San Francisco, CA). In this report, we present the findings of the monitoring for LPS contamination of Hb solutions and document the utility of serial quantitative LPS measurements in developing the ability to successfully produce LPS-free αα Hb.

Materials and Methods

Glassware and reagents

Borosilicate glass test tubes (10 × 75-mm) were obtained from VWR Scientific (San Francisco, CA). Pyrogen-free water and 0.15 *M* (0.15 mol/L) NaCl were obtained from Travenol Laboratories (Deerfield, IL). Human serum albumin (HSA) for intravenous use was obtained from Alpha Therapeutics Corp. (Los Angeles, CA) and IgG for intravenous use from Armour Pharmaceutical Co. (Kankakee, IL).

Culture supplies

We obtained 12 × 75-mm sterile polystyrene plastic culture tubes from Sardstedt Inc. (Newton, NC). Medium (RPMI-1640), L-glutamine, penicillin-streptomycin, and phosphate-buffered saline were obtained from Whittaker Bioproducts, Inc. (Walkersville, MD). Ficoll-hypaque and Histopaque-1077 were obtained from Sigma Chemical Company (St. Louis, MO).

Limulus amebocyte lysates and testing

Amebocyte lysates were prepared from *Limulus polyphemus* (the North American horseshoe crab) by lysis of washed amebocytes in sterile, pyrogen-free water.[17,18] Limuli were obtained from the Department of Marine Resources, Marine Biological Laboratory (Woods Hole, MA). Lysates were stored at 4°C.

We assayed all samples for endotoxin concentration with the Limulus amebocyte lysate (LAL) test, using gelation as the major endpoint.[17,18] Lysate sensitivity was 10 pg per mL for *Escherichia coli* LPS B, 055:B5.

LPS

We obtained *E. coli* LPS B, 055:B5, from Difco Laboratories (Detroit, MI) and used it as the endotoxin standard for both endotoxin assays. A concentration of 1 ng per mL of this *E. coli* endotoxin is equivalent to 4.5 EU (endotoxin units) per mL, referenced to the endotoxin standard EC-5.[19]

Mononuclear cell tissue factor assay for bacterial endotoxin

Human peripheral blood mononuclear cells (MNCs; mean: 12% monocytes, 83-86% lymphocytes, 2-5% neutrophils) were prepared and cultured at 1×10^6 cells per mL as described previously.[20] We incubated MNCs with various preparations of αα Hb or with LPS, in the presence or absence of αα Hb, for 20 hours at 37°C. The MNC cultures then were sonicated and assayed for tissue factor (TF) procoagulant activity by a one-stage coagulation assay, as described previously.[21] Units of TF were established by comparing the clotting times of the cell sonicates with those of dilutions of human brain tissue factor. We defined a clotting time of 30 seconds as equal to 100 units of TF procoagulant activity, as described previously.[22] Cell sonicates with clotting times ≥130 seconds, which equalled the clotting times of the buffer blank (i.e., substrate plasma plus buffer), were judged to have no procoagulant activity.

αα Hb

Human cell-free Hb, crosslinked between the two α chains with bis(3,5-dibromosalicyl) fumarate as described previously,[23] was produced by BRD/LAIR. During a 2-year period, samples were generated from 26 production runs. Also evaluated from many of the runs were samples of starting red cells, partially purified stroma-free hemolysate, and purified cell-free Hb before and after crosslinking and before buffering and concentration of the final products. For experiments in which bacterial endotoxin was spiked in vitro into αα Hb, HSA, or IgG, the protein preparations contained less than 10 pg per mL of endotoxin (referenced to *E. coli* LPS B, 055:B5), as determined by the LAL test.

Results

Sequential monitoring of LPS concentrations during αα Hb production

During a 2-year period, we assayed multiple samples of Hb solutions during the generation and purification of Hb. Samples were defined as negative if LPS concentrations were <10 pg per mL, low-positive if LPS was between 10 pg per mL and 1 ng per mL, and high-positive if LPS was >1 ng per mL. During the early production runs, the majority of Hb samples gave high-positive readings for contamination by LPS (Fig. 1). The high-positive Hb samples were frequently contaminated by ≥100 ng per mL of LPS, as referenced to the *E. coli* standard. For many of the production runs, endotoxin contamination was detected in wash solutions from concentrating tanks or ultrafiltration membranes used for Hb production. Feedback of our findings to the BRD/LAIR production facility, along with the identification of specific sites that were responsible for LPS contamination during the purification of Hb, led to directed efforts to re-establish endotoxin-free conditions. As a result, during the final one-third of the production runs, there were rare high-positive Hb samples and a greatly reduced number of low-positive samples (Fig. 1). Hb samples from the final three production runs (total of 34 Hb samples) all contained <10 pg per mL of LPS, which indicated that endotoxin-free conditions had been achieved and continuously maintained.

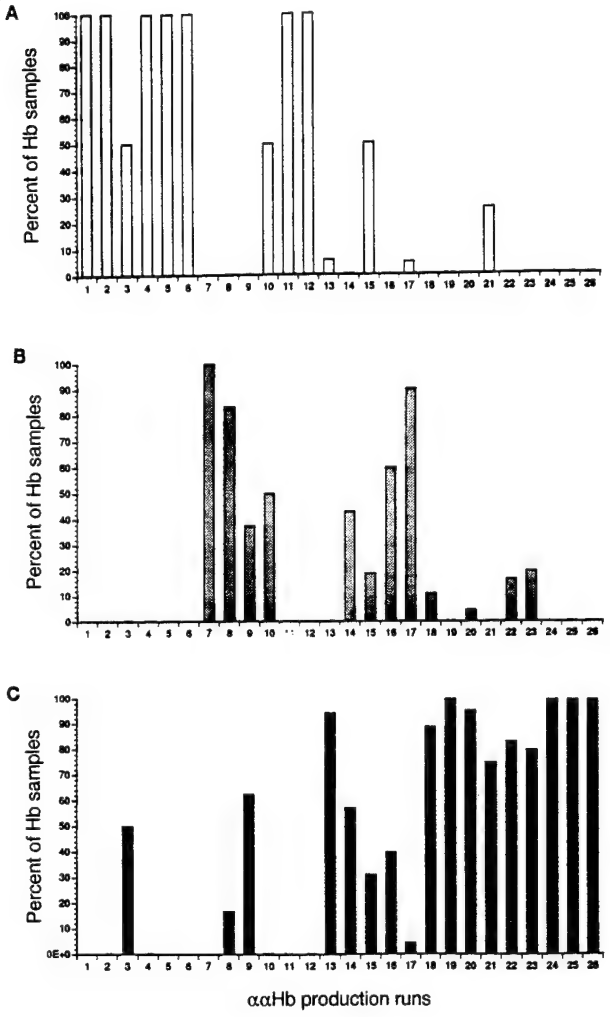

FIG. 1. Endotoxin concentrations in Hb solutions. Hb-containing samples were obtained at multiple steps during the production of αα Hb from each of 26 production runs. From 4 to 15 Hb samples from each run were assayed for LPS concentration by the LAL test. LPS concentrations are characterized as A) high-positive (>1 ng/mL), B) low-positive (10 pg/mL-1 ng/mL), or C) negative (<10 pg/mL).

From each of 14 production runs, five or more Hb-containing samples at various stages of purification were provided for endotoxin assays. Five of these 14 production runs generated one or more high-positive (>1 ng/mL LPS) Hb samples. We examined these five runs to determine if LPS contamination occurred consistently at one step during production of Hb. We compared three major groups of production steps: red cell lysis and filtration of Hb samples to remove stroma (permeates/filtrates); concentration, buffer exchange, and deoxygenation of Hb samples (concentrated Hb); and crosslinking, buffer exchange, reoxygenation, and filtration/concentration to obtain the final product (αα Hb). High-positive Hb samples were distributed among these three groups (Table 1). However, the frequency of contamination with LPS during the final stage (24%) was much higher than that during the initial two stages (5-11%). In some instances, Hb samples with high LPS contamination became low-positive or negative following subsequent ultrafiltration steps (in particular, following 500-kDa membrane ultrafiltrations to remove stroma and aggregated protein). However, in many instances, high-positive Hb samples that had become contaminated by LPS at a step prior to production of the final αα Hb remained high-positive, which resulted in an unacceptable final product. Surprisingly, from nine production runs in which starting red cells (prior to lysis) were provided for assay, two red cell samples contained high levels (>100 ng/mL) of LPS.

Table 1. *LAL-positive samples* at the three major stages of preparation of αα Hb*

	Permeates/ filtrates	Concentrated Hb	αα Hb
Run 13 (n = 20)†	0	0	1
Run 14 (n = 10)	1	0	0
Run 15 (n = 16)	0	2	6
Run 17 (n = 24)	0	0	1
Run 21 (n = 5)	0	0	1

* Hb solutions that contained >1 ng per mL of LPS. Hb-containing samples were obtained from runs with at least five samples available for the determination of LPS concentration.
† Number of Hb-containing samples available from each run for LPS analysis.

Comparison of two independent in vitro assays for LPS concentration

To confirm that the positive LAL assays had been produced by LPS, we assayed 14 Hb samples (including uncrosslinked Hb and αα Hb) for LPS concentration with the LAL test (activation of an invertebrate proteolytic coagulation cascade) and by stimulation of the production of procoagulant activity (TF) from human MNCs. LPS concentrations in the Hb samples encompassed a 5 $\log_{10}$ range (1 pg/mL-100 ng/mL) and were categorized within three broad ranges of LPS contamination: ≤1 ng per mL of LPS, 1 to 100 ng per mL of LPS, and ≥100 ng per mL of LPS. Thirteen of 14 Hb samples were concordantly characterized by the two assay techniques (Table 2).

Enhancement of LPS biologic activity by Hb

The very high LPS concentrations (≥100 ng/mL) that were detected in several of the Hb samples by the LAL test suggested the possibility of an enhancement effect by Hb on the biologic activity of LPS, a known potential phenomenon in this in vitro LPS assay. The concordance of the results of the LAL test and the MNC procoagulant assay strongly suggested that the biologic activity of LPS in the presence of Hb was, in fact, very high for a number of environmentally contaminated Hb samples. Therefore, we investigated the ability of Hb to enhance the biologic activity of added LPS. LPS activation of LAL (Fig.

Table 2. *Comparison of LPS concentration by the LAL* and MNC TF assays*

	MNC TF assay		
	≤1 ng/mL	1-100 ng/mL	≥100 ng/mL
LAL assay			
≤1 ng/mL	10	0	0
1-100 ng/mL	0	0	††
>100 ng/mL	0	0	3

* Fourteen Hb-containing samples each contained demonstrable LPS (>10 pg/mL) according to the LAL test.
† This sample contained 1 ng per mL by the LAL assay and ≥100 ng per mL by the MNC TF assay.

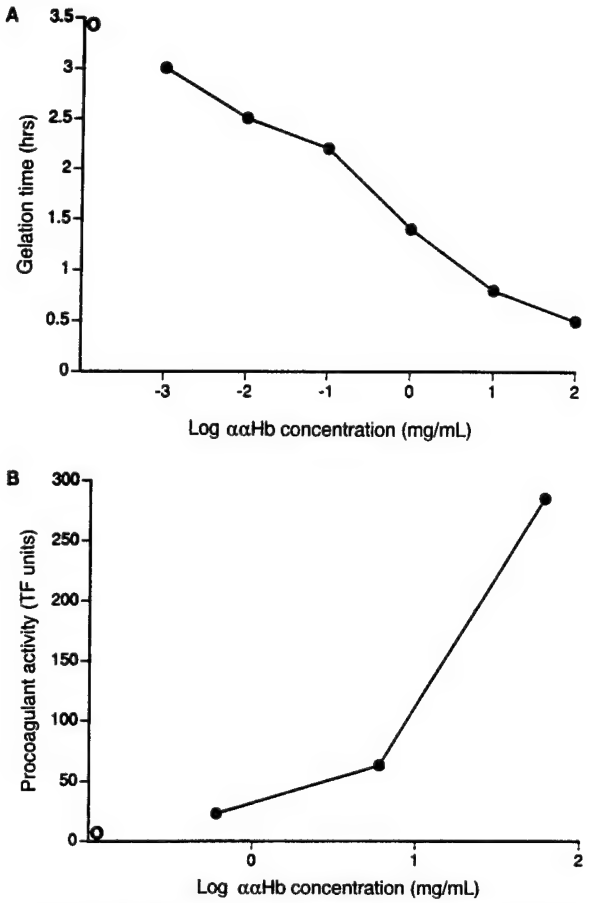

FIG. 2. Enhancement of the biologic activity of endotoxin by Hb. A) Limulus lysate was incubated with *E. coli* LPS (100 ng/mL) at 37°C, in the presence of various concentrations of endotoxin-free ααHb (1 μg/mL - 100 mg/mL Hb) (●). In the absence of added Hb (○), gelation of Limulus lysate by LPS was observed only after 3.5 hours. Gelation time of Limulus lysate was decreased by the presence of Hb in a concentration-dependent manner. This resulted in an apparent increase in the concentration of endotoxin. The ααHb alone contained <10 pg per mL of LPS and did not gel the lysate. B) Human MNCs were incubated with *E. coli* LPS (100 ng/mL) in the presence of various concentrations of ααHb (0.6-60 mg/mL Hb) (●), and TF generated by each LPS/Hb mixture was then measured. The contribution of ααHb alone, which at the maximal concentration of 60 mg per mL contained 0.5 ng per mL of LPS, to the total TF generated by the MNCs was subtracted, at each concentration, from the measured total. Enhanced generation of TF activity by LPS was stimulated by Hb in a concentration-dependent manner. LPS alone (100 ng/mL) (○) generated 13 units of TF in the MNC assay; concentrated Hb alone (60 mg/mL) generated 95 units of TF.

2A) and generation of MNC procoagulant activity (Fig. 2B) were enhanced, in a concentration-dependent fashion, by ααHb. Further investigation of this phenomenon, using the LAL test, demonstrated that the minimum concentration of ααHb required to produce enhancement of LPS activity was variable and ranged from 0.05 to 1.0 mg/mL for different preparations of ααHb (data not shown).

Comparative effects of serum proteins on LPS biologic activity

The observation that ααHb enhanced LPS activity led to the possibility that LPS contamination of Hb solutions might

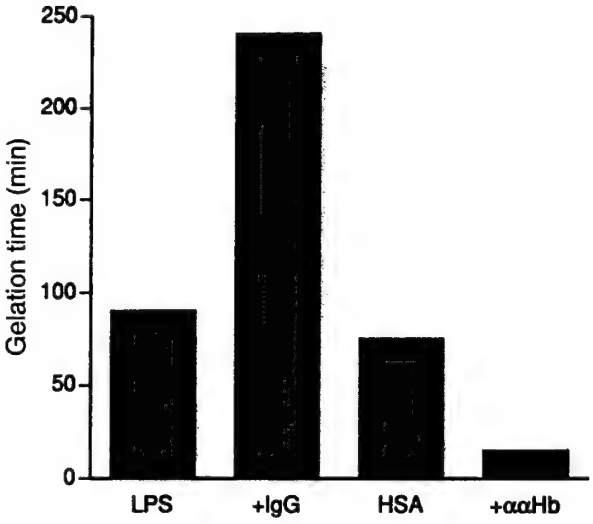

FIG. 3. Effect of serum proteins on the biologic activity of endotoxin. Limulus lysate was incubated with *E. coli* LPS (500 ng/mL) alone or in the presence of 5 mg per mL of endotoxin-free IgG, HSA, or ααHb. Gelation time of Limulus lysate, compared to that of LPS in NaCl, was increased 267 percent by IgG, which indicates the inhibition of LPS biologic activity by IgG. In contrast, gelation times were decreased 25 percent by HSA and 83 percent by ααHb, which indicates slight and substantial enhancements of LPS activity by HSA and ααHb, respectively.

represent a particularly important contribution toward in vivo toxicity. To determine whether the enhancement effect was a special property of Hb, we compared LPS biologic activity in the presence of two other protein solutions (HSA and IgG) commonly administered at high concentrations and relatively large volumes. Whereas ααHb greatly increased LPS biologic activity over that in NaCl, HSA resulted in a minimal enhancement, and IgG resulted in a substantial inhibition (Fig. 3).

Discussion

Our detection of high concentrations of LPS in some preparations of ααHb supports previous experimental evidence[9,14,15] that LPS contamination of Hb preparations can limit the feasibility of administration of this blood substitute to humans. We have demonstrated that Hb preparations can be significantly contaminated by environmental LPS, with several final ααHb solutions containing ≥100 ng per mL of LPS (referenced to *E. coli* LPS B, 055:B5). An adult patient requiring Hb for resuscitation would likely require at least one-tenth of his or her blood volume, that is, approximately 500 mL, of Hb solution. If ααHb were contaminated with ≥100 ng per mL of LPS, initial plasma LPS levels would be at least 10 ng per mL. The administration of sufficient LPS (250,000 ng) to produce LPS plasma concentrations of this magnitude would be significantly toxic and would constitute a serious risk. For instance, approximately 300 ng of *E. coli* LPS given to human volunteers elicited fever, tachycardia, nausea, and myalgias.[24] Plasma LPS levels above 10 ng per mL in patients with sepsis have been associated with death,[25] and plasma LPS levels

above 1 ng per mL during *E. coli* infusion into primates similarly were associated with death.[26] Finally, the infusion of *E. coli* LPS into pigs (resulting in steady-state LPS levels of 1 ng/mL) caused the activation of coagulation, characterized by the utilization of kallikrein and prothrombin, and the stimulation of plasmin activity.[27] Physiologic responses to a certain concentration of infused LPS may be augmented by the presence of Hb, which in our study enhanced LAL activation and MNC TF production, as is consistent with the previous demonstration that Hb produces synergistic toxicity with LPS in vivo.[10]

A major component of the severe pathologic sequelae of endotoxemia is the stimulation of release of MNC cytokines, including tumor necrosis factor,[24,28-30] interleukin 1,[25,31,32] interleukin 6,[25,31,32] and the expression of cell-associated TF, a potent procoagulant activity.[20] Because of the large volumes of Hb solution that would be infused into patients receiving αα Hb as a blood substitute, the endotoxemia resulting from the infusion of LPS-containing solutions might elicit many of the cytokine-mediated features of septic shock. Furthermore, recipients of αα Hb probably would already be experiencing endotoxemia because of trauma or gastrointestinal tract ischemia and thus may have increased sensitivity to the infusion of additional endotoxin.

We have shown that Hb samples that strongly activated LAL also strongly stimulated human MNCs to produce TF. This assay was selected to confirm the LAL assay results because of its sufficient sensitivity,[20] in contrast to chemical and immunologic methods for detection of LPS. The validity of LAL-determined LPS concentrations in the ng per mL range in plasma recently has been demonstrated by the use of a gas chromatography-mass spectrometry method specific for neisserial LPS,[33] further confirming the ability of the LAL assay to measure biologically significant LPS in protein-containing solutions. In the MNC TF assay employed in the present study, several preparations of Hb that were LAL negative were, correspondingly, not stimulatory. Others have apparently demonstrated an intrinsic property of Hb to elicit MNC procoagulant activity,[34] although the levels of procoagulant activity detected by those investigators appear to be very low. However, our study did not provide evidence that endotoxin-free (≤10 pg/mL LPS) Hb had an intrinsic ability to stimulate MNC procoagulant activity.

We have demonstrated that focal breaks in techniques designed to maintain endotoxin-free conditions can occur at any step during the purification of Hb. Because of the complexity of the Hb purification and crosslinking processes, it is clearly advantageous to sequentially monitor multiple Hb samples and buffers for the presence of LPS during production runs. This is feasible because of the simplicity and the quantitative characteristics of the LAL assay. The high level of sensitivity of the LAL test, which can be further augmented by recognition of pregelation changes in lysate[18] or by chemical enhancement of weak chromogenic activities,[35] ensures that solutions described as LAL-negative are essentially endotoxin-free. In addition, the ability of the LAL assay to determine concentrations of the biologic activity of contaminating LPS relative to that of a standard LPS allows the establishment of the magnitude of contamination. Although data concerning LAL testing of the physical components of the manufacturing process were not presented in this report, it was possible in several instances to document that a specific component of the production apparatus (e.g., a particular ultrafilter) was not endotoxin-free prior to its contact with Hb. Determination of the exact site at which LPS has been introduced into an Hb sample allows a practical, directed effort to remove the endotoxin from the system.

As a result of detailed monitoring of LPS in washes of the production apparatus and in Hb solutions at multiple points during purification of Hb, production of endotoxin-free αα Hb by BRD/LAIR became routine. This is a critically important result, given the toxicity-related limitations of Hb infusion that have been observed previously in human and animal trials.[5-14] A remaining concern is our incomplete understanding of the ability of purified cell-free Hb to alter cardiovascular status. However, recently reported clinical trials of Hb solutions[36-40] demonstrated that the development of Hb as a blood substitute has made significant progress. Our evidence that αα Hb can be made sufficiently endotoxin-free that MNCs and coagulation cascades are not activated, despite the ability of Hb to enhance LPS biologic activity, provides additional confidence that αα Hb may be an acceptable transfusion alternative in the future.

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Liver Hemodynamics During Portal Venous Endotoxemia in Swine

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The acute hemodynamic response of the liver to portal endotoxemia was measured in six isoflurane anesthetized pigs in which volume support was used to maintain normal cardiac output. After baseline monitoring, bacterial endotoxin (LPS) was infused over 1 hr into a mesenteric vein at a rate of 1 $\mu g \cdot kg^{-1} \cdot hr^{-1}$, and monitoring was continued for 1 hr postinfusion. Peak vasoconstriction occurred during LPS infusion in both the hepatic artery (resistance ↑ 349% of baseline, $P < 0.05$) and the liver's portal circulation (resistance ↑ 159% of baseline, $P < 0.05$). Increased vascular resistance was also detected in lung (↑ 433% of baseline) and intestine (↑ 130% of baseline) at the midpoint of the LPS infusion. The non-splanchnic circulation, defined for our analysis as all of the peripheral circulation except the portal and hepatic arterial circulation, generally exhibited little change in vascular resistance during LPS infusion. LPS was incompletely cleared by the liver, but secondary clearance by the lung prevented large increases in the LPS concentration of arterial blood. During the first hour postinfusion, the systemic vascular resistances subsequently decreased to near normal in all vascular beds, with the exception of the liver's portal circulation. A sustained and secondary increase in vascular resistance of the liver's portal circulation and portal vein pressure occurred during the first hour after LPS infusion. We conclude that most of the vasoconstriction in the acute response to portal endotoxemia occurs in the liver and lung, organs directly exposed to elevated levels of endotoxins.

Key words: sepsis, hepatic blood flow, endotoxin, portal vein, pig

INTRODUCTION

Septic shock is a late, and often fatal, complication of trauma and critical illness. The extent to which septic shock results from a systemic response to whole bacteria or endotoxin remains controversial [1]. Infusion of the lipopolysaccharide (bacterial endotoxin, LPS) of cell walls of gram-negative bacteria into experimental animals and man can, however, be used to mimic most of the early and sustained cardiovascular alterations of clinical sepsis [2–4]. Although vascular entry of bacterial LPS may occur via several potential routes, recent studies support the hypothesis that gut ischemia leads to an alteration in the gut's mucosal barrier and that, as a result, both bacteria and LPS can enter the circulation via the portal circulation and intestinal lymphatics [5–8]. The presence of detectable endotoxin in the plasma of patients correlates positively with bacteremia, clinical sepsis, and increased mortality [1,9].

The liver and lung are in an anatomical position to provide the secondary defenses that limit the extent of systemic endotoxemia after translocation of gastrointestinal tract endotoxin. These organs have large intrinsic macrophage populations that may effectively clear most bacteria and LPS that enter their circulations under normal circumstances. Activation of macrophages stimulates release of various cytokine and inflammatory mediators that can have profound effects on blood flow and microvascular permeability. The pulmonary response to venous infusion of LPS is well-described and consists of an initial hypertensive phase followed by a more sustained phase of increased microvascular permeability [3,10,11]. Less is known about the response of the liver to portal endotoxemia.

Gut blood flow typically decreases with circulatory shock, particularly during experimental septic shock [12]. Such reductions in gut blood flow are large, and out

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of proportion to the decreases in cardiac output. Without concurrent data on gut blood flow and portal pressures, it is difficult to determine if the reduced flow results from vasoconstriction in the intestine or liver. The relative contributions of the liver's arterial and portal circulations to this flow reduction have not been directly measured.

The present studies were designed to better characterize the early hemodynamic response of the liver to portal endotoxemia. Toward that end, we measured pressures and flows in the hepatic artery and portal vein during and after a 1-hr portal vein infusion of LPS in anesthetized pigs. Our goal was not to study hypodynamic endotoxic shock, but rather to study hepatic and splanchnic hemodynamics without the confounding phenomenon of systemic hypoxia due to a low flow state. Therefore, volume support was used as needed to maintain cardiac output. The Limulus lysate assay was used to measure LPS in blood sampled from the hepatic vein and the aorta.

MATERIALS AND METHODS

Animal Preparation

Twelve Yorkshire male pigs, 35–50 kg, were fed standard pig chow and housed in large pens while awaiting study. Nine animals were used for LPS experiments, but only six survived. Three animals served as controls in which saline was infused instead of LPS. Prior to each experiment, food was withheld for a period of 12 hr, but free access to water was provided. On the day of study, the animals were initially sedated with an intramuscular injection of 0.4 mg/kg acepromazine, 0.1 mg/kg atropine, and 20 mg/kg of ketamine. A 20-gauge ear vein IV cannula was inserted and an infusion of 500 ml of sterile pyrogen-free 0.9% saline was administered preoperatively. After intubation, inhalation anesthesia was maintained with 1–2% isoflurane, 50% O_2, and the remainder air. After induction of anesthesia, animals were placed on an operating room table covered with a heating blanket. The animals were periodically checked for jaw tone and lid twitch responses; anesthetic levels were adjusted as needed. All studies were terminal and complied with the NIH guidelines for animal anesthesia and surgery.

Multiple sterile pyrogen-free vascular catheters were inserted into the superior vena cava and thoracic aorta via neck vessels. These catheters included a 12-gauge IV extension tube in the left external jugular vein; a Swan-Ganz pulmonary arterial catheter inserted via the left internal jugular vein; an aortic catheter inserted via the left common carotid artery; and a left hepatic venous catheter inserted via the right external jugular vein. The latter catheter's placement was confirmed by palpation during laparotomy. A continuous IV infusion of lactated Ringer's solution was initiated and adjusted as necessary for maintenance of baseline cardiac output during the surgery and experiment.

The abdomen was opened in the lower midline and the bladder was exposed. A Foley catheter suprapubic cystostomy was performed and the urethra was ligated. The upper abdominal organs were then exposed using a bilateral subcostal incision, and the small bowel was packed out of the surgical field. The portal triad was identified, and the portal vein and hepatic artery were dissected free of the common bile duct that was left intact and unobstructed. The portal vein was encircled with a 10-mm Doppler transit time flow probe (Transonics, Ithaca, NY). A large lymph node overriding the inferior aspect of the portal vein was frequently removed to facilitate the placement of this probe. In a similar manner, the animal's hepatic artery was then encircled with a 4-mm flow probe.

After the flow probes were placed, vascular access to the portal circulation was achieved with two staggered sterile pyrogen-free pediatric feeding tubes. The first tube was inserted into the distal splenic vein and secured at the cutaneous exit site after being threaded distally into the portal vein for pressure monitoring. A second catheter was placed into the superior mesenteric vein in an area just distal to the second portion of the duodenum and secured via a purse-string suture. This second catheter remained within the superior mesenteric vein and was used for the infusion of endotoxin or control vehicle. A distance of 4–5 cm separated the tips of the two portal catheters.

Experimental Protocol

After the completion of surgery, a 3-hr experimental protocol was undertaken. Nine animals were subjected to LPS infusion, while three animals served as controls. Initially, a 1-hr baseline period was permitted to allow stabilization of vascular pressures and organ blood flows. Following the baseline period, the animals received a continuous 60-min infusion of bacterial endotoxin (*Escherichia coli* LPS, 055:B5, Difco Laboratories, Detroit, MI) through the superior mesenteric venous catheter. A dosage of endotoxin equal to 1 μg/kg animal weight was suspended in 0.9% pyrogen-free saline at a final concentration of 2 μg/ml immediately prior to administration. All infusions were performed with a Harvard pump apparatus through sterile pyrogen-free IV tubing. Control animals received saline alone, without endotoxin. Following the infusion, a 1-hr postinfusion observation period took place. Both groups received lactated Ringer's in an attempt to maintain cardiac outputs near normal. At the end of this time, the animals were killed with an overdose of saturated potassium chloride solution while still under full general anesthesia.

Measured Variables

Blood samples and hemodynamic measurements were obtained during the 3-hr experimental period. Vascular

pressures were measured and recorded using Gould P23 pressure transducers and a Gilson stripchart recorder system. When coupled to previously placed vascular catheters, this system allowed measurement of mean aortic arterial pressure (MAP), heart rate (HR), central venous pressure (CVP), portal venous pressure (PVP), mean pulmonary arterial pressure (PAP), and pulmonary arterial wedge pressure (PWP). Readings were recorded every 10 min during the experimental period. All pressure readings were taken at end-expiration. Blood gas determinations were used to adjust ventilation to maintain normal CO_2.

Blood sampling was performed at 10-min intervals with the exception of arterial blood gas analysis that was performed at 20-min intervals. An initial 6-ml blood sample was collected in a sterile pyrogen-free heparinized syringe immediately after insertion of the aortic arterial catheter and used as baseline plasma for several assays. During the experimental period, 4-ml blood samples were simultaneously withdrawn from the aortic, portal venous (splenic), and left hepatic venous catheters every 10 min and placed in heparinized sterile pyrogen-free tubes for centrifugation and separation of plasma. Plasma was transferred to a second set of pyrogen-free tubes and stored at −70°C until assayed for LPS.

Vascular blood flows were also quantified at 10-min intervals. Blood flow in the portal vein ($\dot{Q}_{pv}$) was measured with a 10-mm transit time flow probe while hepatic arterial blood flow ($\dot{Q}_{ha}$) was measured using a 4-mm probe. Both probes were connected to a two-channel flowmeter (Transonics, Ithaca, NY). Cardiac output (CO) was measured using the thermodilution technique with a cardiac output computer (Baxter-Edwards, Santa Ana, CA). All determinations were made at end-expiration, and the mean of at least two injections for each 10-min period was recorded.

LPS Assay

In 7 of the experiments (5 LPS and 2 controls), we measured LPS levels in hepatic venous and arterial blood. *Limulus* amebocyte lysate was prepared from the washed amebocytes of *Limulus polyphemus* by hypotonic lysis, as described previously [13]. Endotoxin concentrations in plasma were determined by a modification of a previously described method [14,15]. Heparinized plasma was diluted fourfold with pyrogen-free 0.15 M NaCl and heated at 60°C for 30 min to neutralize inhibitors. Fifty μl heated diluted plasma then was incubated with 50 μl *Limulus* amebocyte lysate for 4 hr at 37°C, with visual inspections at 15 min intervals for LAL activation. Samples were graded for flocculation, increased viscosity, and gelation, as previously described [15]. Standard curves were established by spiking a series of concentrations of LPS into endotoxin-free pig plasma. The same *E. coli* LPS used for infusion was used for these standards. Experimental plasma endotoxin concentrations were calculated by comparison of the rates of gelation (i.e., rates of progression from flocculation to increased viscosity to gelation) produced by the samples, with the rates of gelation established by the standard curves. The *Limulus* amebocyte lysate used for these studies had a lower limit of LPS detection of 1 pg/ml of the reference endotoxin in pig plasma, and could discriminate between plasma endotoxin concentrations over a 4-log range (1 pg/ml–10 ng/ml). Plasma samples were assayed in duplicate. When LPS concentrations above background were detected, the levels were confirmed by assay of 10-fold and 100-fold diluted samples.

Calculated Variables

Measurement of the flows and pressures indicated above allows us to calculate the vascular resistance of several components of the peripheral circulation using standard formulas for adding series and parallel resistance, as needed. For purposes of analysis in this paper, we define the splanchnic circulation as both the portal circulation and the liver's arterial circulation. The remainder of the systemic circulation is defined as the nonsplanchnic circulation.

It is realized that the entire splanchnic blood flow is greater than portal and hepatic arterial blood flow, but this definition allows us to evaluate changes quantitatively in the vascular resistance of the intestine and liver, and compare these changes to those in the remainder of the peripheral circulation. The schematic in Figure 1 defines the variables we calculated with the following equations:

Systemic vascular resistance (R_{sys}) = (MAP − CVP)/CO.

Pulmonary vascular resistance (R pul) = (PAP − PWP)/CO.

Liver's hepatic arterial vascular resistance (R ha) = (MAP − CVP)/$\dot{Q}_{ha}$.

Liver's portal vascular resistance (R pv) = (PVP − CVP)/$\dot{Q}_{pv}$.

Intestinal vascular resistance (R int) = (MAP − PVP)/$\dot{Q}_{pv}$.

Splanchnic vascular resistance (R_{spl}) = 1/[1/(R int + R pv) + 1/R ha].

Nonsplanchnic vascular resistance (R_{nspl}) = 1/[1/R_{sys} − 1/R_{spl}].

Statistics

Data are reported in text and tables as mean ± 1 SD for evaluation of variance, and reported in figures as mean ± 1 SEM for comparison of the two sample populations. We used the Friedman analysis of variance test to

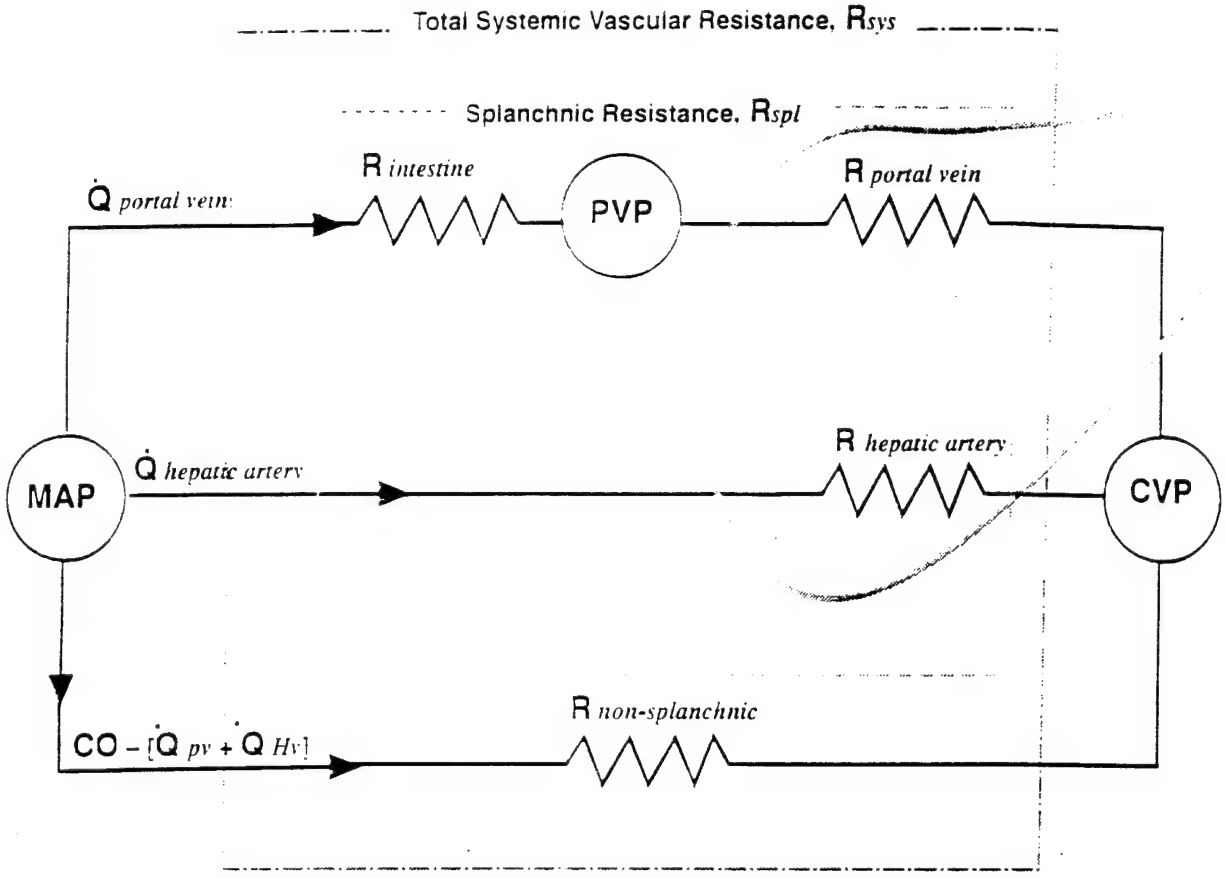

Fig. 1. Schematic of the measured pressures (P), blood flows (Q), and calculated resistances (R) of the peripheral circulation. Variables defined as in text.

determine whether variables during and after LPS infusion were significantly different ($P < 0.05$) than baseline.

RESULTS

Hemodynamics

Surgical preparation of each animal required approximately 3 hr, and was associated with a blood loss of 100–200 ml. Temperature was well maintained during the study, using placement of warmed fluid bags next to the pig. Baseline hemodynamic readings taken 1 hr post-surgery indicated a stable mean arterial blood pressure, central venous pressure and cardiac output. This required substantial fluid support of 60–100 ml · kg^{-1} · hr^{-1} due to the extensive nature of the dissection. High-level fluid support was continued during LPS infusion and data collection to maintain cardiac output near baseline throughout the protocol. Three animals in the LPS group died during LPS infusion and their data are not included in the tables or figures; thus, n = 6.

Blood pressures and blood flows for both groups are shown in Figures 1–3. Baseline mean arterial blood pressure was approximately 70 mm Hg in both groups. Central venous pressures averaged 3–5 mm Hg, while mean pulmonary artery wedge pressure was approximately 8 mm Hg. Mean pulmonary artery pressures were within a range of 14–17 mm Hg. Baseline cardiac output averaged approximately 5 L/min in both groups. Mean hepatic arterial flow was 200–300 ml/min. Portal venous flows averaged 700–950 ml/min. During the baseline period, total hepatic blood flow represented approximately 20–25% of cardiac output. Four to 6 percent of cardiac output was via hepatic arterial flow and 15–20% was via portal venous flow.

Control experiments were performed to determine the physiological effects of the 3-hr surgical preparation and the subsequent 3 hr of anesthesia and monitoring. The figures show that the hemodynamic variables of the 3 control pigs were stable over the monitoring period. Because variables were relatively constant in control animals, we chose not to perform additional control experiments. Therefore, we did not perform statistical analysis to compare the control and LPS groups; rather, we performed statistical analysis of the LPS animals, making comparisons between baseline measurements, and during and after LPS infusion.

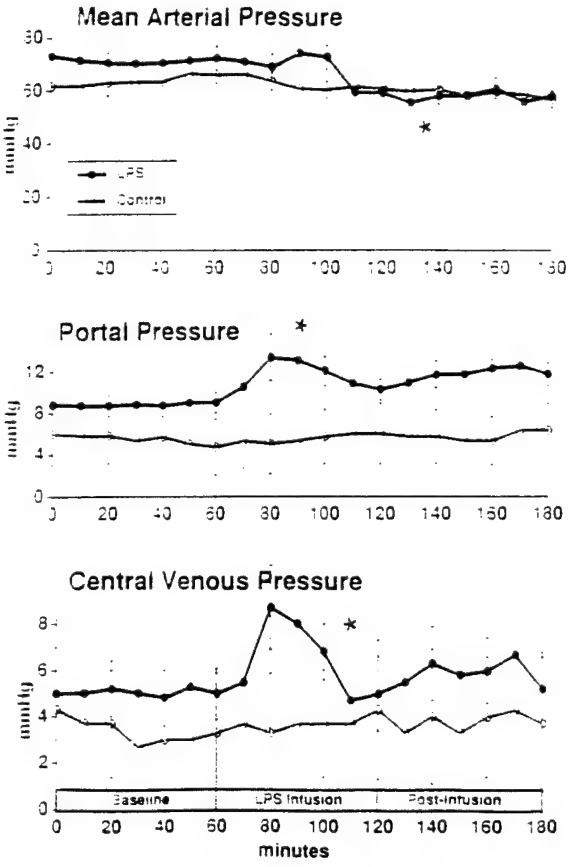

Fig. 2. Mean arterial pressure, portal vein pressure and right atrial (central venous) pressure during and after a 60-min portal infusion of LPS at 1 $\mu g \cdot kg^{-1} \cdot hr^{-1}$ in anesthetized swine. Asterisk denotes statistically significant difference ($P < 0.05$) of data during a 60-min period compared to 60-min baseline period. The fall in arterial pressure during the post-LPS infusion period was statistically significant ($P < 0.05$). A peak increase of portal pressure ($P < 0.05$) and central venous pressure ($P < 0.05$) occurred 20 min into the LPS infusion. Number of pigs: LPS (n = 6); control (n = 3).

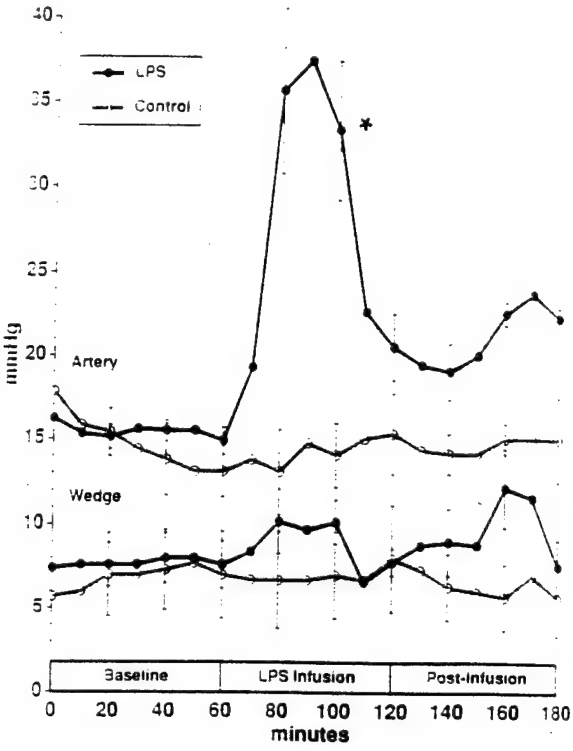

Fig. 3. Pulmonary artery pressure and pulmonary wedge pressure during and after an LPS infusion. Asterisk denotes statistically significant difference ($P < 0.05$) of data during a 60-min period compared to 60-min baseline period. The increase in pulmonary artery pressure was statistically significant ($P < 0.05$), while changes in wedge pressure were not ($P = 0.07$).

Only slight changes in hemodynamics were observed within the first 10 min of endotoxin infusion into the portal vein. At 10 min, however, portal vein pressure, pulmonary artery and wedge pressure had begun to increase ($P < 0.05$) with peak responses recorded at 20–30 min into the infusion (Figs. 2, 3). There was a slight fall in the mean cardiac output during LPS infusion, but the change did not reach statistical significance ($P = 0.06$). Continued fluid infusion resulted in a full return to baseline values before the end of the LPS infusion (Fig. 4). Cardiac output was sustained at baseline to the end of the study (Fig. 4). At 20 min into the LPS infusion, profound alterations in pulmonary pressures and hepatic blood flows took place (Figs. 3, 4). The three animals that died did so during the LPS infusion due to apparent hypoxemia and cardiac arrest. Pulmonary hypertension was always observed during LPS infusion, $P < 0.05$ (Fig. 3).

Mean pulmonary artery pressures rose to 30–40 mm Hg, $P < 0.05$, between 20–40 min into LPS infusion, and remained maximally elevated for 10 min. This represented a 250% increase over baseline. By 40 min of infusion, pulmonary hypertension had begun to abate and mean pulmonary pressure was 130–160% of baseline during the post infusion period which was not significantly different than baseline. Central venous (CVP) and portal venous (PVP) pressures also rose in a similar trend during the acute response phase. Although CVP fell to baseline levels postinfusion, PVP remained slightly elevated during most of the postinfusion period so that the portal-central vein pressure gradients increased to 7–8 mmHg over the latter third of each study, compared to a baseline gradient of 4 mmHg. Figure 5 shows that mean vascular resistance was greater in both the total systemic ($P = 0.12$) and pulmonary circulation ($P < 0.05$) during infusion. During the 1-hr postinfusion, systemic vascular resistance decreased to below baseline ($P < 0.05$), while mean pulmonary vascular resistance was slightly above baseline.

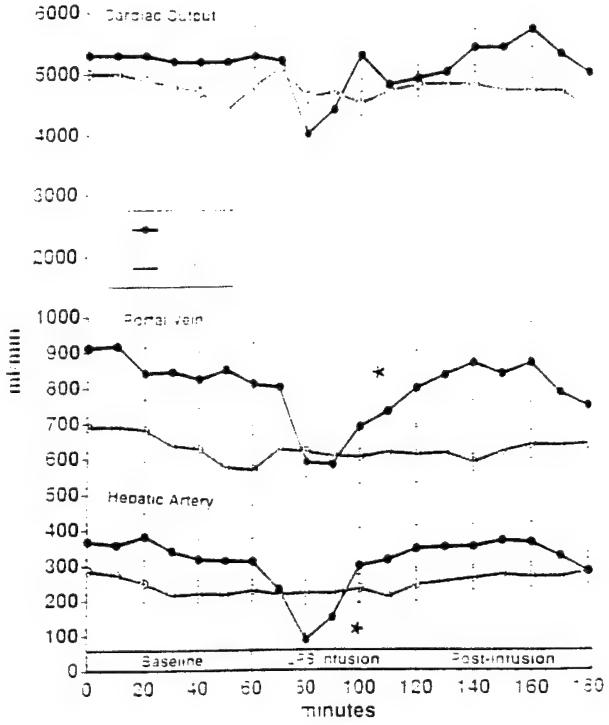

Fig. 4. Total systemic blood flow (cardiac output) and blood flow through the portal vein and hepatic artery during and after an LPS infusion. Asterisk denotes statistically significant difference ($P < 0.05$) of data during a 60-min period compared to a 60-min baseline period. The declines in blood flow of the portal vein and hepatic artery at 20–40 min into the LPS infusion were statistically significant, while the decline in cardiac output was not significant ($P = 0.06$).

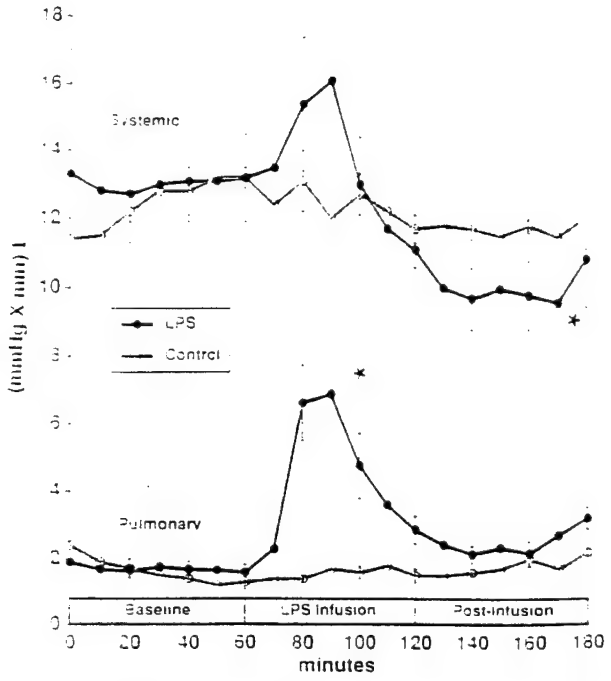

Fig. 5. Vascular resistance of the peripheral vascular bed and the pulmonary circulation during and after an LPS infusion. Asterisk denotes statistically significant difference ($P < 0.05$) of data during a 60-min period compared to 60-min baseline period. The total systemic vascular resistance exhibited an initial increase ($P = 0.12$) and then fell after LPS infusion ($P < 0.05$). Pulmonary vascular resistance increased 2- to 4-fold during LPS infusion ($P < 0.05$).

Concurrent with the early acute pulmonary response, all measurements of blood flow (CO, $\dot{Q}_{ha}$, and $\dot{Q}_{pv}$) decreased as a result of portal venous endotoxemia (Fig. 4). Cardiac output fell from 5.5 ± 1.3 to 4.1 ± 1.1 liters per min; $\dot{Q}_{ha}$ fell from 327 ± 175 to 97 ± 40 ml/min, $P < 0.05$; and $\dot{Q}_{pv}$ fell from 839 ± 311 to 592 ± 150 ml/min, $P < 0.05$. The 30% fall in portal venous flow was paralleled and matched by a 25% decrease in CO. The greater than 70% fall in $\dot{Q}_{ha}$, however, suggests specific hepatic vasoconstriction in addition to the decrease in total CO. Hepatic arterial vascular resistance increased to 349% of baseline during this period (Fig. 6). Hepatic arterial flow and vascular resistance returned to baseline before the end of the endotoxin infusion. This pattern was not seen in the portal circulation, in which there was a smaller, but sustained, elevation in portal venous vascular resistance (Fig. 6).

Table 1 shows vascular resistances in the total splanchnic circulation (R_{spl}) calculated from the combined resistance of the total portal circulation and hepatic arterial circulation. For comparison, the calculated vascular resistance for the remainder of the nonsplanchnic peripheral circulation (R_{nspl}) is also shown. Non-splanchnic peripheral resistances exhibited a mild initial increase which was not statistically significant; thereafter, mean values decreased to below baseline. Splanchnic vasoconstriction was sustained during LPS infusion, but fell to baseline levels after infusion.

The level of total blood flow through the portal circulation is set by the relatively larger resistance and vascular pressure drop in the intestine. Figure 7 compares the time course of the changes in the vascular resistances of the intestine and liver. The increased resistance in the intestine during LPS infusion returned to normal after infusion, while the vascular resistance of the liver's portal circulation remained elevated and exhibited a second maximum at 60 min postinfusion. This resulted in sustained portal vein hypertension. The reductions in gut blood flow during LPS infusion are primarily due to intestinal vasoconstriction, since intestinal vascular resistance and portal blood flow quickly and completely returned to near-normal levels after LPS infusion.

Liver Clearance of Endotoxin

Endotoxin (LPS) concentrations were measured in plasma sampled from aorta, hepatic vein, and portal vein.

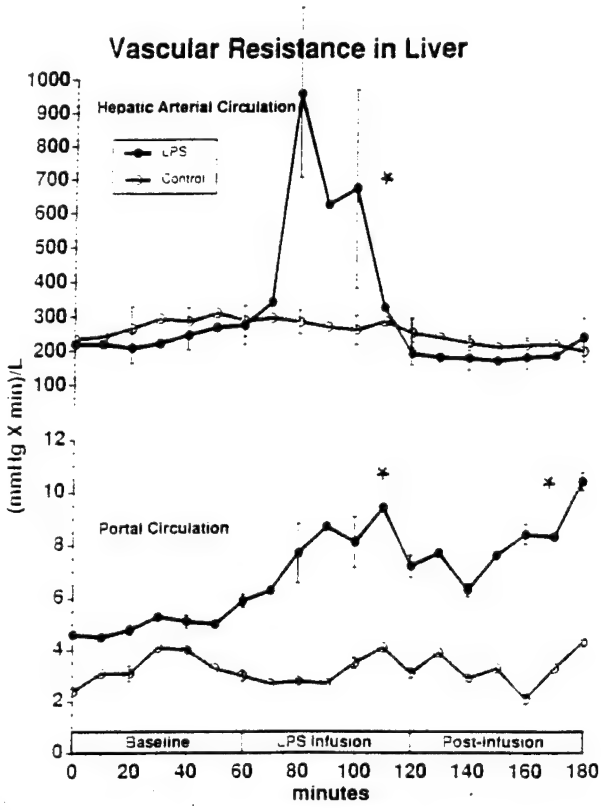

Fig. 6. Vascular resistances of the hepatic arterial circulation ($P < 0.05$) and the portal venous circulation ($P = 0.06$) increased during LPS infusion. Asterisk denotes statistically significant difference ($P < 0.05$) of data during a 60-min period compared to 60-min baseline period. Resistance of the portal circulation exhibited a secondary rise after LPS infusion ($P < 0.05$).

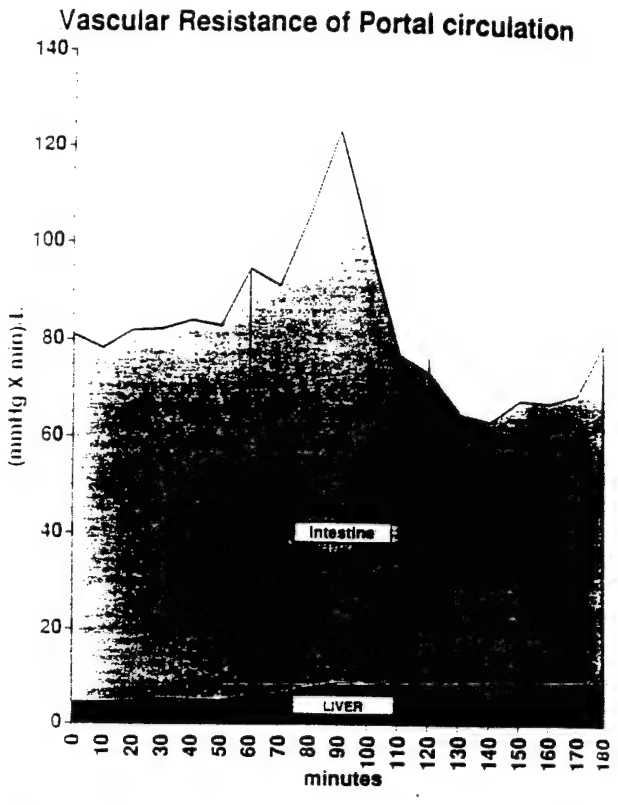

Fig. 7. The relative contributions of intestine and liver to the series vascular resistances of the portal circulation during and after LPS infusion.

TABLE I. Splanchnic and Nonsplanchnic Peripheral Vascular Resistance (mm Hg · min · L^{-1})†

	R_{spl}	R_{nspl}
Baseline	56.0 ± 18.8	16.6 ± 4.5
20 min of LPS infusion	93.3 ± 39.4*	18.6 ± 5.6
40 min of LPS infusion	72.4 ± 26.4	15.9 ± 3.9
30 min postinfusion	45.2 ± 14.4	12.9 ± 3.4
60 min postinfusion	56.3 ± 25.0	13.8 ± 4.2

†R_{spl}, splanchnic vascular resistance; R_{nspl}, nonsplanchnic peripheral vascular resistance.
*$P < 0.05$ vs. baseline.

Values from the portal vein varied widely during LPS infusion, probably because of some streaming of the LPS dose during mesenteric vein infusion and inadequate mixing prior to portal vein sampling. LPS concentrations are reported in plasma samples from aorta and hepatic vein obtained at 10 min intervals from 2 control and 5 experimental animals (Fig. 8). Except for contamination of hepatic vein samples during the early points of the preinfusion period for Pig F, data points were technically adequate for these 7 experiments. LPS concentrations in plasma from the control pigs were usually < 0.5 pg/ml, although isolated peaks as high as 8.0 pg/ml were measured during the 3-hr experiment. There were no differences between LPS levels measured in samples from the aorta or hepatic vein in the control animals.

In animals that received LPS, elevated levels of LPS (>5 pg/ml) were detected with peak concentrations ranging from 10 pg/ml to 1,000 pg/ml. In each of these samples, the elevated LPS concentrations were confirmed with repeat assays by both the standard assay procedure and in plasma which had been further diluted, as described under Materials and Methods. In each pig, peak levels were detected during the period of LPS infusion in blood samples obtained from the hepatic vein (Fig. 8). Samples from the aorta generally exhibited little change from baseline levels. Increased levels of endotoxin in the hepatic vein were not constant during the 1-hr LPS infusion despite the infusion rate being constant. There were peaks of 20- to 40-min duration in each animal. Levels of LPS in the hepatic vein had returned toward the normal range before the end of the LPS infusion period, suggesting that the ability of the liver to clear LPS was increased in response to exposure to LPS. The hepatic vein data show that LPS is entering the central venous circulation, while the substantially lower levels of

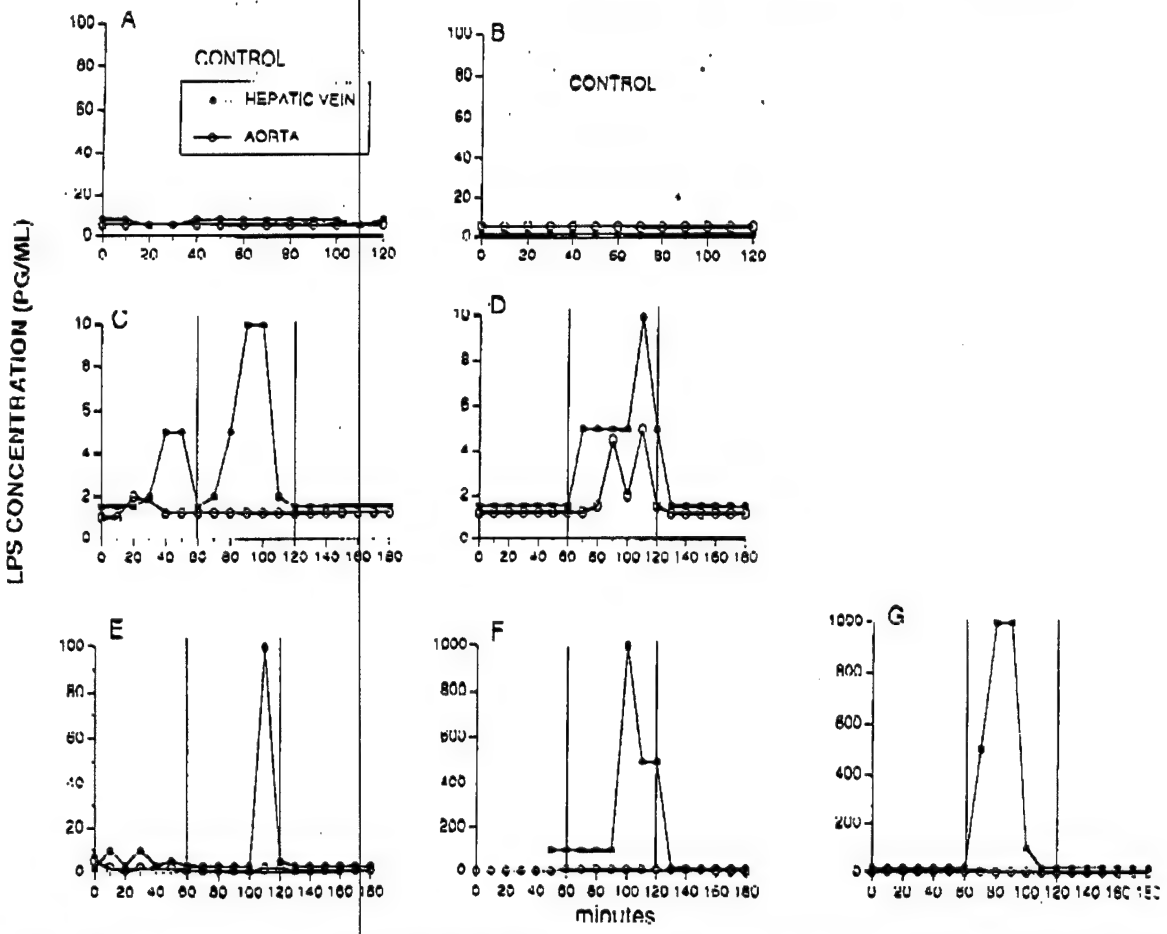

Fig. 8. LPS concentrations in plasma. Blood was sampled from the aorta and hepatic vein before (0–60 min), (60–120 min), and after (120–180 min) a 1-hr infusion of 0.9% NaCl in 2 control pigs (A,B), and LPS (1 μg/kg) in 5 experimental pigs (C–G). LPS infusions are indicated by the two vertical lines in C–G. Plasma samples were diluted and heated, as described in Materials and Methods, and LPS concentrations in the treated plasma samples were determined by the Limulus amebocyte lysate (LAL) test. Vertical axis scales are variable.

LPS in the aorta suggest additional LPS clearance has been performed by the lung.

DISCUSSION

Nearly all previous studies of experimental endotoxemia have used peripheral intravenous infusion. However, clinical endotoxemia is more likely to occur due to entry of LPS into the portal circulation. Splanchnic ischemia, bowel obstruction, GI surgery and sepsis have all been associated with the translocation of bacteria and their cell wall products across the gastrointestinal mucosal barrier [1,5,12]. The specific hemodynamic responses of the liver to portal venous endotoxemia have not been well defined. Because differences may exist in the systemic response to various sites of initiation of endotoxemia, this study attempted to quantify the specific vascular response to endotoxin administered through the portal vein. Our model allowed quantification of the hemodynamic changes within the liver's two circulations.

Many types of circulatory shock, including sepsis, reduce mesenteric blood flow out of proportion to changes in cardiac output [12]. Navaratnam et al. demonstrated that total mesenteric blood flow was decreased in an ovine model of endotoxemia [16]. Schrauwen and Houvenaghel found similar decreases in mesenteric arterial blood flow with central venous bolus injections of LPS in anesthetized piglets [17]. These studies are in agreement with our finding that the initial response to portal venous endotoxemia was a profound vasoconstriction in both the intestinal and hepatic vascular beds 20 min following the beginning of infusion of LPS.

The intense initial vasoconstriction in the gut following portal endotoxemia may be mediated by thromboxane.

The initial pulmonary hypertension that occurs after central injections of LPS is associated with increases in plasma thromboxane A2 [11,18]. Administration of cyclooxygenase inhibitors can greatly attenuate the pulmonary hypertension [19]. Thromboxane synthetase blockade also prevents the increased mesenteric vasoconstriction found after burn injury [20]. Thromboxanes are synthesized by stimulated macrophages, and thus, the vasoconstriction in both the liver and lung may be caused by activation of each organ's macrophages.

There was a clear difference in magnitude between the initial vasoconstriction occurring in the arterial and portal circulations of the liver. Hepatic arterial flow was reduced to a minimum value of less than 30% of baseline and was associated with a threefold increased vascular resistance. However, both blood flow and resistance recovered postinfusion in the hepatic arterial circulation. Portal blood flow fell as much in absolute units (~200 ml/min), but this was a proportionally smaller reduction to 60% of its baseline value. Blood flow in both circulations returned to baseline after LPS infusion, but the vascular resistance of the hepatic portal circulation remained elevated and exhibited a second postinfusion increase.

The sustained increase in the vascular resistance of the liver's portal circulation may be due to vasoconstriction, but also could be a consequence of parenchymal and endothelial cellular swelling and microvascular margination of activated and sequestered neutrophils. Circulatory shock can cause both cellular edema of liver parenchyma and endothelial cells and neutrophil margination in the microcirculation [21,22]. Another explanation for increased resistance could be secondary to vascular congestion and increased microvascular hematocrits and red cell aggregation, which would increase apparent vascular resistance by affecting viscosity.

Our data suggest that the initial hemodynamic responses to endotoxemia primarily occur in the microvascular beds that are directly downstream from the source of endotoxin. The one exception to this was the initial vasoconstriction in the intestine that occurred without high LPS levels in aortic blood. Elevated plasma LPS entered the circulations of both the liver and lungs, while relatively lower levels, similar to those measured in control animals, entered other organs. The circulations of the liver and lung provide in-line series clearances of LPS. This double organ clearance may be critical for the protection of other organs from the toxicity of LPS. The cytokine cascade, beginning with the release of tumor necrosis factor, and followed by release of various interleukins, activates other cellular defense systems [23]. The ability of bacterial endotoxins to activate multiple inflammatory pathways and to cause system-wide damage may play an important role in the link between septic shock and multiple organ failure [24,25].

There was a substantial range for peak LPS levels in blood sampled from the hepatic vein. This likely results from the large number of variables involved in LPS clearance, including hepatocyte function, the state of the reticuloendothelial system, lipoprotein levels, and rapidly changing levels of acute phase and normal LPS-binding proteins. Similarly, highly variable LPS concentrations in blood are commonly reported in studies of septic patients.

Peaks were generally present for only 20–40 min of the 60-min infusion period. The magnitudes of the peak increases were much larger than the reductions in hepatic blood flow, indicating a real reduction in the liver's LPS extraction during the infusion. This suggests that hepatic LPS clearance mechanisms may have been overwhelmed early in the infusion period, but then were able to recover or were up regulated.

The initial large increases in pulmonary and hepatic vascular resistance were paralleled by a smaller 10–20% increase in total systemic vascular resistance (R_{sys}). All of the increase in R_{sys} was attributed to the splanchnic circulation. However, R_{sys} quickly fell to below baseline even before the LPS infusion ended. The mean vascular resistance of the nonsplanchnic organs was less than baseline during most of the LPS infusion and also after the infusion (Table I). This is in contrast to the splanchnic circulation in which mean vascular resistance generally remained at baseline or higher. Reduced peripheral vascular resistance is a common finding in clinical hyperdynamic sepsis. Our data suggest that the splanchnic circulation may not participate in the loss of vascular tone during sepsis.

The acute hemodynamic alterations in response to portal endotoxemia can be of sufficient magnitude to result in death, as demonstrated by 3 of the 9 experimental animals dying during LPS infusion. This early vasoconstrictive phase, however, most evident in the pulmonary and hepatic arteries, rapidly peaked and returned to near normal despite continuation of the LPS infusion.

CONCLUSIONS

In summary, portal endotoxemia in anesthetized swine leads to profound but brief periods of vasoconstriction in the intestine, lung, and liver. Significant vasoconstriction did not occur in the nonsplanchnic peripheral circulation; on the contrary, mean resistance decreased. The liver and lung circulations provided effective serial clearance of LPS. Following LPS infusion, only the portal circulation of the liver had a sustained increase in vascular resistance.

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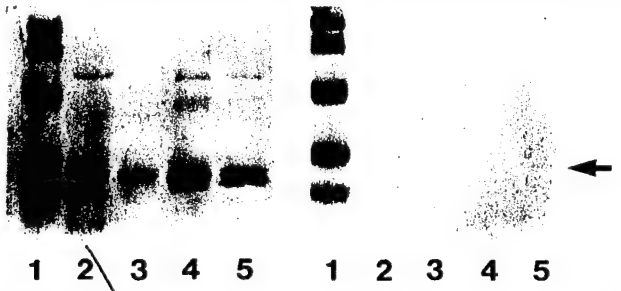
All animal experiments described in this manuscript were conducted in adherence with the NIH Guidelines for the Use of Experimental Animals.



SDS-gel electrophoresis stained with Amido black (left) and nitrocellulose transfer
tted with LAS34 monoclonal antibody against Hb A_{1c} (right). Lane 1, Molecular
ndard; lane 2, Hb A_{1c}; lane 3, non-A_{1c} glycohemoglobin; lane 4, Hb A_{1c}; lane 5,
single immunoreactive band (arrow) consistent with hemoglobin A_{1c} monomer is
es 2 and 4 on the right.

bin may spuriously elevate absorbance values, samples should
ed against Hb A_0 subjected to the same procedure. Results are
d as moles of HMF/mole of hemoglobin, which should have a
t least 1 mol/mol of β-chain monomer for Hb A_{1c} and may have
mol of tetramer for non-A_{1c} glycohemoglobin. However, HMF
nay not be quantitative.[11]

blotting

y specific monoclonal antibodies reactive with glucose in keto-
kage with the N-terminal valine residue or with the lysine amino
hemoglobin can be used for validation of glycohemoglobin prepa-
nd confirmation of their purity. The antibodies must be specific
elevant glycated epitope in hemoglobin, because cross-reactivity
glycated) hemoglobin or with other (glycated) proteins may con-
sults. Two antibodies described in the literature seem to meet
teria.[20,23] Hemoglobin A_{1c}, but not Hb A_{1a}, Hb A_{1b}, Hb A_0, or
glycohemoglobin, will immunoreact with monoclonal antibodies
e raised against a glycated synthetic peptide that comprised sev-
no acid units corresponding to the N terminus of the hemoglobin
The peptide was glycated *in vitro* at the N-terminal valine position
pled to an immunogenic carrier. Another monoclonal antibody
raised against Hb A_{1c} isolated from normal human erythrocyte
hows specific immunoreactivity with Hb A_{1c} (unpublished), and
of reactivity with Hb A_0 or with hemoglobin glycated in non-A_{1c}
s (Fig. 5). Non-A_{1c} glycohemoglobin, but not Hb A_0, Hb A_{1a},

noules, M. T. Marchesi, and W. Holen, U.S. Pat. 4,727,036 (1988).

Hb A_{1b}, or Hb A_{1c} (unless it also is glycated at a lysine residue) will immunoreact with monoclonal antibodies raised against non-A_{1c} glycohemoglobin purified from human erythrocyte lysates.[20]

Acknowledgments

This work was supported in part by grants from the Ben Franklin Fund of the Commonwealth of Pennsylvania and from the Department of Human and Health Services (HL 44767).

[6] Measurement of Endotoxin Levels in Hemoglobin Preparations

By ROBERT I. ROTH and JACK LEVIN

Endotoxin (lipopolysaccharide, LPS) is a glycolipid component of the outer portion of the cell wall of gram-negative bacteria (Fig. 1). Bacterial endotoxin, which is responsible for the fever, disseminated intravascular coagulation, hypotension, and cardiovascular shock that frequently accompany gram-negative septicemia, produces biological and pathophysiological effects at very low concentrations (in the picogram per milliliter range), even in the absence of living bacteria. Because endotoxemia is associated with substantial morbidity and mortality, FDA guidelines require that sufficiently low endotoxin levels be documented to allow human use of parenteral drugs or medical devices. This review will focus on the use of the *Limulus* amebocyte lysate test to measure endotoxin levels in hemoglobin preparations, but will also describe its more general applications for the detection of endotoxin in protein solutions and blood.

Limulus Amebocyte Lysate Test

The *Limulus* amebocyte lysate (LAL) test is an *in vitro* biological assay for endotoxin, based on endotoxin activation of the horseshoe crab coagulation cascade.[1] Lysates of the amebocytes (blood cells) of the North American horseshoe crab *Limulus polyphemus* (or the Japanese horseshoe crab *Tachypleus tridentatus*) contain an endotoxin-activated proteolytic coagulation cascade[2] that is exquisitely sensitive to bacterial endotoxins. This *in vitro* assay has replaced the older rabbit pyrogen test for endotoxin,

[1] J. Levin and F. B. Bang, *Bull. Johns Hopkins Hosp.* **115,** 265 (1964).
[2] N. S. Young, J. Levin, and R. A. Prendergast, *J. Clin. Invest.* **51,** 1790 (1972).

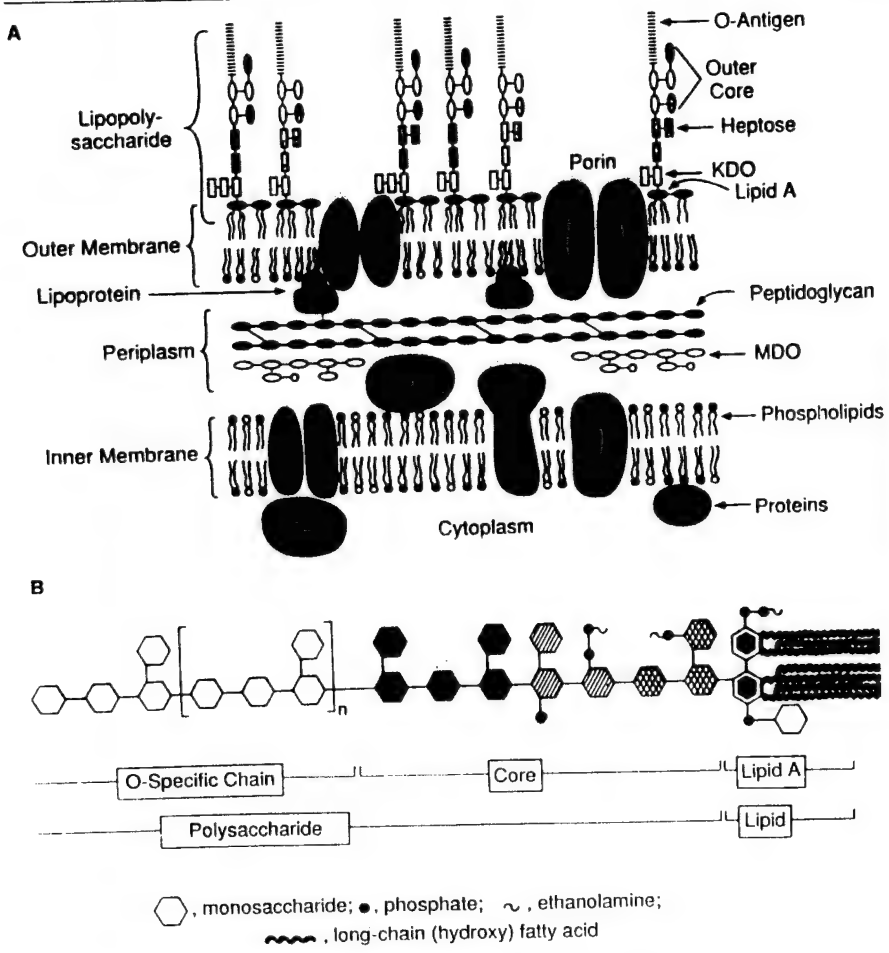


FIG. 1. (A) Schematic representation of the *E. coli* envelope. Lipopolysaccharide is located in the external portion of the outer membrane. Ovals and rectangles depict sugar residues. Circles represent the polar heads of phospholipids. MDO, Membrane-derived oligosaccharides; KDO, 3-deoxy-D-*manno*-octulosonic acid. Reproduced, with permission, from the *Annual Review of Biochemistry*, Vol. 59, © 1990 by Annual Reviews Inc. (B) Schematic structure of lipopolysaccharide (*Salmonella*). Repeating units of the O-specific chain plus core oligosaccharide form the hydrophilic polysaccharide portion of lipopolysaccharide. The hydrophobic lipid A portion consists of a β-1,6-linked D-glucosamine disaccharide backbone substituted with phosphoryl, phosphorylethanolamine, arabinose, and fatty acyl groups. Reproduced with permission from E. Th. Rietschel *et al.*, *Scand. J. Infect. Dis.* (Suppl.) **31**, 10 (1982).

and currently is used to monitor endotoxin in intravenous fluids, parenteral drugs, and medical devices. The LAL test is the most sensitive assay currently available for the detection of endotoxin and can detect crude, native LPS associated with the bacterial cell wall (Fig. 1A), highly purified lipopolysaccharide, and lipid A (Fig. 1B). The minimum concentration of endotoxin detectable by LAL reagents is typically 5–10 pg/ml, although specific batches of LAL may be sufficiently sensitive to quantify endotoxin in the femtogram per milliliter range. Sensitivity also depends on the biological activity of a specific endotoxin.

Activation of the LAL coagulation cascade by endotoxin leads to the subsequent activation of a trypsinlike serine protease, clotting enzyme, which cleaves the clottable protein coagulogen. Proteolytic activation of coagulogen to coagulin by clotting enzyme is followed by polymerization of coagulin, thus resulting in formation of a gel. The rate of this process is directly proportional to the endotoxin concentration,[1,3] and the rate of LAL activation can be used to quantify endotoxin concentrations over a very wide range (e.g., from 1 pg/ml to at least 100 ng/ml).[1] An example of the relationship between rates of gelation and endotoxin concentration is shown in Fig. 2 using a lysate with a lesser sensitivity range.

Three different end points have been utilized for the LAL assay (Table I). First, gelation (formation of a solid gel)[3] is the basis of the gel-clot, pass/fail test that is frequently used for manufacturing and other commercial applications. In this assay, a small volume of test sample (e.g., 50 μl) and a similarly small volume of lysate (or lyophilized reagent) are incubated together at 37° for a fixed amount of time, typically 1 hr, and observed for generation of a solid, translucent gel (Table I and Fig. 3). The lysate has been characterized previously to determine the minimal concentration of LPS required to generate a solid gel within the selected time (cutoff concentration), and therefore the LPS concentration in an unknown sample can be determined to be either greater or less than the gelation cutoff. Samples that gel the lysate can be retested after a series of dilutions to obtain further quantification. In addition, the actual time of gelation can be utilized to provide a more precise measurement of endotoxin concentration. Furthermore, by the recognition of earlier clotting stages (flocculation and increased viscosity)[1] it is possible to use the LAL gelation reaction as a quantitative assay that not only becomes more sensitive but can measure endotoxin over a wider range of concentrations. Second, spectrophotometric measurement of increasing turbidity (related to coagulin polymerization)[3] is a quantitative, automated version of the LAL test that

[3] J. Levin and F. B. Bang, *Thromb. Diath. Haemorrh.* **19**, 903 (1970).
[4] J. F. Cooper, J. Levin, and H. N. Wagner, Jr., *J. Lab. Clin. Med.* **78**, 138 (1971).

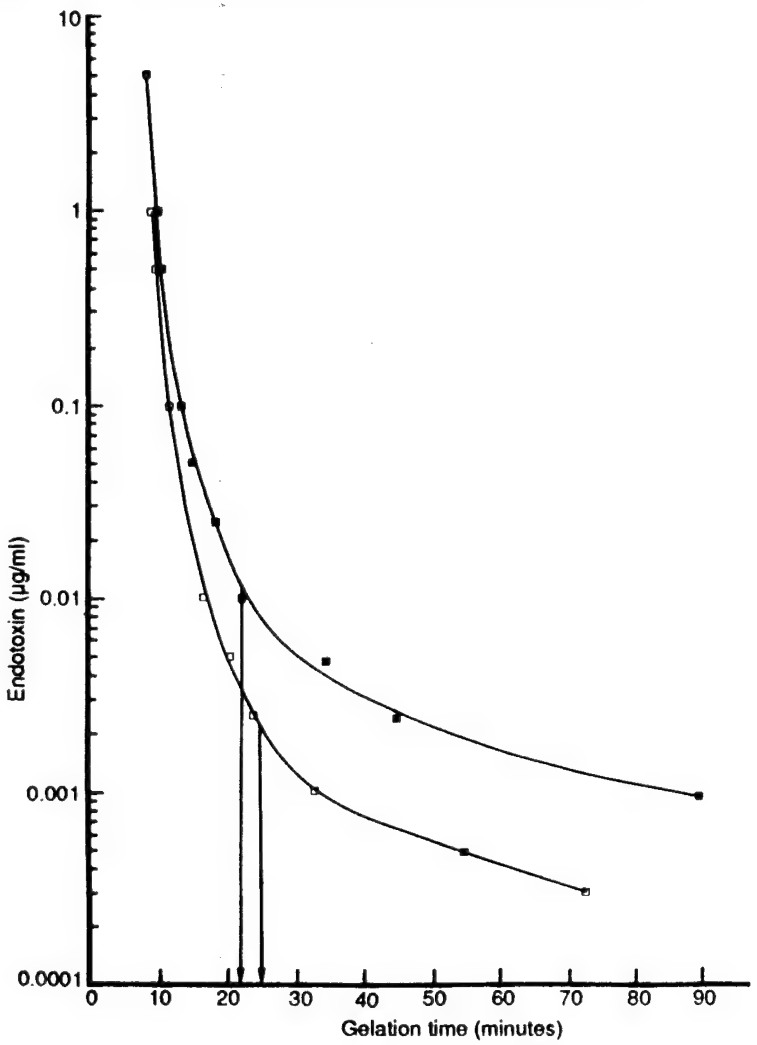


FIG. 2. Gelation of *Limulus* amebocyte lysate by *E. coli* (■) and *Klebsiella* (□) endotoxins. Incubation mixtures containing 0.1 ml *Limulus* lysate and 0.1 ml endotoxin were incubated at 37°. Gelation times are proportional to endotoxin concentration over a wide range of endotoxin concentrations [from J. F. Cooper, J. Levin, and H. N. Wagner, Jr., *J. Lab. Clin. Med.* **78,** 138 (1971), with the permission of the publisher].

makes use of the characteristic lag period and subsequent rate of increase in light scattering, which are endotoxin concentration-dependent (Table I and Fig. 4). An automated method for determination of endotoxin concentration by measurement of increased viscosity also has been described.[5] Third, spectrophotometric measurement of the rate of generation of clotting enzyme activity, which is also endotoxin concentration dependent and is typically measured with a chromogenic or fluorogenic substrate for the protease,[6] is the basis for an automated, quantitative assay for the protease that generates the subsequent turbidity (Table I and Fig. 5). If the chromogenic LAL assay is utilized, additional sensitivity for lower concentrations of LPS can be achieved with derivatization of the chromogen with diaminocinnamaldehyde.[7] The concentration of endotoxin in a sample is quantified by determining its LAL reactivity with any of the above described methods and relating LAL activation to that produced by a series of concentrations of a standard endotoxin (i.e., a standard curve).

TABLE I

PERFORMANCE OF *Limulus* AMEBOCYTE LYSATE TEST FOR QUANTIFICATION OF ENDOTOXIN CONCENTRATION IN SAMPLES OF SFH

1. To ensure that endotoxin is not introduced into the SFH sample to be assayed, heat all glassware (180°, 3–4 hr), or utilize sterile plasticware previously documented to be endotoxin-free (<10 pg/ml endotoxin, based on currently utilized standards, is usually satisfactory)
2. Transfer 50-μl aliquots of SFH into either endotoxin-free borosilicate glass test tubes or sterile microtiter plate wells
3. Add 50 μl of *Limulus* amebocyte lysate (LAL), the sensitivity of which has been previously determined utilizing a common reference lipopolysaccharide (e.g., *E. coli* 055:B5 or EC-5) for the standard curve
4. Incubate mixtures at 37° and monitor end points of LAL activation as follows:
   (a) For gel-clot assay, observe the mixture for the presence of a solid gel at a standard time, e.g., 1 hr
   (b) For gelation assay with increased sensitivity, observe the mixture at 15- to 30-min intervals for pregelation changes (weak flocculation, heavy flocculation, increased viscosity) and ultimately for gelation time (if endotoxin concentration is sufficient)
   (c) For turbidimetric assay, monitor increase in light scattering nephelometrically or monitor increase in spectrophotometric absorbance (305 nm)
   (d) For chromogenic assay, preincubate the SFH–LAL mixture for a standard time at 37°, e.g., 10–30 min, then add 100–200 μl of chromogenic substrate S-2222 or S-2423 (Kabi Vitrum, Molndal, Sweden), 0.25–0.5 m*M*, pH 7.5–8.0, and monitor amidolytic activity at 405 nm. Absorbance at 405 nm can be determined at a fixed time after addition of chromogenic substrate, e.g., 10–30 min, or kinetic analysis of the rate of increase in absorbance at 405 nm can be performed. Detection of amidolytic activity at 405 nm may be optimized by conversion of hemoglobin to carboxyhemoglobin, to minimize interference by oxyhemoglobin or methemoglobin in the spectrophotometric detection of the enzymatic activity.[30]

[5] R. Homma, Y. Takada, I. Karube, K. Kimura, and H. Muramatsu, *Anal. Biochem.* **204,** 398 (1992).

[6] P. Friberger, M. Knos, and L. A. Mellstam, *in* "Endotoxins and their Detection with the *Limulus* Amebocyte Lysate Test" (S. W. Watson, J. Levin, and T. J. Novitsky, eds.), p. 195. Alan R. Liss, New York, 1982.

[7] R. I. Roth, J. Levin, and S. Behr, *J. Lab. Clin. Med.* **114,** 306 (1989).

FIG. 3. Gelation of *Limulus* amebocyte lysate by bacterial endotoxin. *Limulus* lysate initially is a clear, colorless fluid (left). After addition of endotoxin and incubation at 37°, the lysate becomes an opaque, solid gel (right) [from J. Levin, *in* "Blood Cells of Marine Invertebrates" (W. Cohen, ed.), p. 153, Alan R. Liss, New York, © copyright 1985. Reprinted with permission of Wiley-Liss, a division of John Wiley and Sons, Inc.].

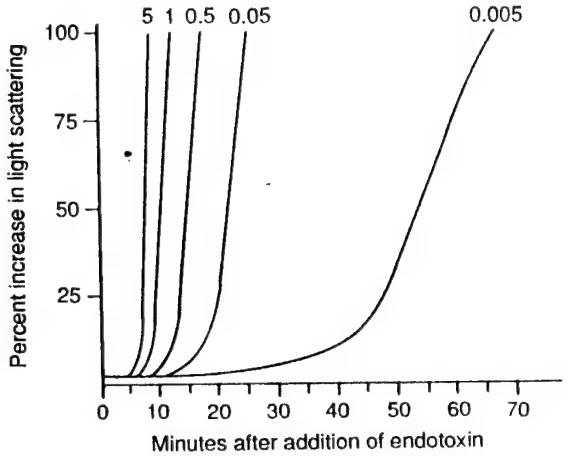


FIG. 4. Increase in turbidity of *Limulus* lysate after addition of bacterial endotoxin. After 0.1 ml *E. coli* endotoxin (0.005–5 μg/ml) was added to 0.9 ml *Limulus* lysate, the mixtures were incubated at 37° and light scattering (turbidity) was measured photofluorometrically. The endotoxin concentration in each incubation mixture is indicated at the top of each curve. At low endotoxin concentrations, there is a substantial lag period followed by a gradual increase in light scattering. At higher concentrations of endotoxin, there is a concentration-dependent decrease in the lag period and increase in the rate of increased turbidity [from J. Levin, P. A. Tomasulo, and R. S. Oser, *J. Lab. Clin. Med.* **75**, 903 (1970), with the permission of the publisher].

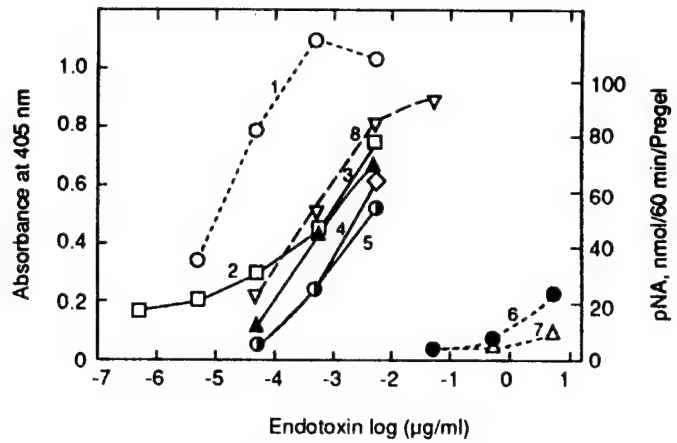


FIG. 5. Increase in amidase activity in *Limulus* lysate after addition of bacterial endotoxin. Preparations of *E. coli* endotoxins (1–5) and *E. coli* polysaccharides (6–8) were added to *Limulus* lysate, followed by the addition of the chromogenic substrate *tert*-butoxycarbonyl-Val-Leu-Gly-Arg-*p*-nitroaniline. Amidase activity (cleavage of the substrate by *Limulus* clotting enzyme) was quantified by determining the absorbance at 405 nm by free *p*-nitroaniline (pNA). Bacterial endotoxins produce a concentration-dependent increase in absorbance at 405 nm, whereas polysaccharides are many orders of magnitude less reactive [from T. Harada-Suzuki, T. Morita, S. Iwanaga, S. Nakamura, and M. Niwa, *J. Biochem.* (*Tokyo*) **92**, 793 (1982), with the permission of the authors].

Determination of endotoxin concentration in a sample with the LAL test is rapid (typically completed within 1–2 hr) and reproducible [coefficients of variation (CV) for replicate samples using common commercial LAL reagents are ±10%]. The results are generally in good agreement with the standardized *in vivo* assay for endotoxin, the rabbit pyrogen test, and with many other (although less sensitive) *in vitro* assays for endotoxin.[4,8,9] Comparison of *Limulus* amebocyte test assays for endotoxin, performed in our laboratory, with rabbit pyrogen test assays of five preparations of stroma-free hemoglobin* has demonstrated that for three hemoglobin preparations containing ≥100 pg/ml endotoxin according to the LAL test *in vitro*, fever was produced *in vivo*, whereas fever was not elicited by two similarly purified hemoglobin preparations that contained ≤10 pg/ml endotoxin.

* Stroma-free hemoglobin (SFH) designates purified hemoglobin that contains negligible amounts of erythrocyte membrane material or other cytosolic components.

[8] J. van Noordwijk and Y. de Jong, *J. Biol. Stand.* **4**, 131 (1976).

[9] P. A. Tomasulo, *in* "Biomedical Applications of the Horseshoe Crab (Limulidae)" (E. Cohen, F. B. Bang, J. Levin, *et al.*, eds.), p. 293. Alan R. Liss, New York, 1979.

Contribution of Endotoxin to *in Vivo* Toxicity of Hemoglobin

Variably pure solutions of hemoglobin have been reported to produce pathophysiological effects when infused into humans. Hypertension and bradycardia have been commonly observed[10,11] and a decrease in glomerular filtration rate and renal plasma flow has been described.[12] Mild prolongations of the partial thromboplastin time also have been described.[11] In some animal studies, preparations of hemoglobin have been shown to produce fever, disseminated intravascular coagulation with resultant thrombosis, and ischemic parenchymal damage.[13,14] Toxicity was due in part to the presence of environmental LPS and in part to the presence of stromal phosphatidylethanolamine and phosphatidylserine, which were shown to produce death in rabbits, in contrast to other tested phospholipids.[13] Some of these changes were observed following administration of hemoglobin reported to be endotoxin free.[14] Similarly, hemoglobin contaminated with membrane phospholipids or LPS caused, *in vitro*, the release of procoagulant activity (tissue factor) and thromboxane from cultured human endothelial cells.[15] Furthermore, major pathological sequelae have been observed after infusion of hemoglobin, in the absence of detectable LPS or phospholipids, and despite the absence of abnormalities in tests of blood coagulation.[16] However, in other animal studies, hemoglobinemia produced by infusion of hemolyzed blood was not associated with laboratory evidence of disseminated intravascular coagulation or with parenchymal organ damage.[17]

Although endotoxin contamination appears to be responsible for some of the *in vivo* toxicity of hemoglobin, there is also evidence that hemoglobin enhances the toxicity of LPS. A dose of bacteria (*Escherichia freundii*), that when administered either alone or in combination with intact red blood cells caused no mortality in dogs, resulted in a 100% mortality when administered in conjunction with a solution of hemoglobin produced by hemolysis of red blood cells.[18] The mechanism of death was believed to be blockade of the reticuloendothelial system with hemoglobin, with

[10] W. R. Amberson, J. J. Jennings, and C. M. Rhode, *J. Appl. Physiol.* **1,** 469 (1949).
[11] J. P. Savitsky, J. Doczi, J. Black, and J. D. Arnold, *Clin. Pharmacol. Ther.* **23,** 73 (1978).
[12] J. L. Brandt, N. R. Frank, and H. C. Lichtman, *Blood* **6,** 1152 (1951).
[13] M. Feola, J. Simoni, P. C. Canizaro, R. Tran, and G. Raschbaum, *Surg. Gynecol. Obstet.* **166,** 211 (1988).
[14] D. H. Marks, T. Cooper, and T. Makovec, *Mil. Med.* **154,** 180 (1989).
[15] M. Feola, J. Simoni, D. Fishman, R. Tran, and P. C. Canizaro, *Artif. Organs* **13,** 209 (1989).
[16] C. T. White, A. J. Murray, J. R. Greene, D. J. Smith, F. Medina, G. T. Makovec, E. J. Martin, and R. B. Bolin, *J. Lab. Clin. Med.* **108,** 121 (1986).
[17] J. I. Spector, C. B. Wilson, and W. H. Crosby, *Am. J. Pathol.* **74,** 567 (1974).
[18] M. S. Litwin, C. W. Walter, P. Ejarque, and E. S. Reynolds, *Ann. Surg.* **157,** 485 (1963).

resultant activation of blood coagulation by bacterial endotoxin and production of the generalized Shwartzman reaction. Synergism also was demonstrated in a study in which the combination of SFH and endotoxin produced a 50–100% mortality rate in recipient rabbits at doses of endotoxin and SFH that did not cause death when administered alone.[19] Paradoxically, the incubation of ferrous iron ($FeSO_4$) with bacterial endotoxin prevented the 50% mortality and production of the generalized Shwartzman reaction that had been produced in rabbits by the administration of control preparations of endotoxin.[20] *In vitro*, either SFH or LPS stimulated the generation of procoagulant activity (tissue factor) by both human and rabbit mononuclear cells, with the combination of SFH and *Escherichia coli* LPS producing an additive effect.[21] In these studies, stimulation of procoagulant activity was associated with heme rather than globin.

In summary, it appears that hemoglobin may have inherent pathophysiological effects, some of which may become more manifest if hemoglobin is administered to an already unstable, physiologically compromised recipient. Under such circumstances, e.g., shock or damage to the colon, it would not be surprising that the potential toxicity of hemoglobin would be increased if preparations of hemoglobin are contaminated with bacterial endotoxins or the recipient of hemoglobin is experiencing endotoxemia. In addition, the potential toxicities of hemoglobin and LPS may be synergistic.

Quality Control Monitoring of Hemoglobin Production

LAL testing has been employed for quality control monitoring of the production of chemically modified [bis(3,5-dibromosalicyl) fumarate cross-linked] stroma-free hemoglobin (DBBF-SFH), as described previously.[22] An abbreviated flow scheme for purification of SFH is presented in Fig. 6; results of LAL testing of the production apparatus (prior to hemoglobin production) and of the hemoglobin product at various stages of its purification are presented in Fig. 7. An evaluation for endotoxin with the LAL test is recommended at each step of the production process, because contamination of hemoglobin by environmental endotoxin can occur at any stage. Commonly, endotoxin contamination of hemoglobin preparations occurs as the result of a localized loss of nonpyrogenic (i.e.,

[19] C. T. White, A. J. Murray, D. J. Smith, J. R. Greene, and R. B. Bolin, *J. Lab. Clin. Med.* **108,** 132 (1986).
[20] A. Janoff and W. Zweifach, *J. Exp. Med.* **112,** 23 (1960).
[21] D. J. Smith and R. M. Winslow, *J. Lab. Clin. Med.* **119,** 176 (1992).
[22] R. M. Winslow, K. W. Chapman, and J. Everse, *Biomater. Artif. Cells, Immobilized Biotechnol.* **19,** 503 (1991).

Add 0.9% NaCl to red blood cells
↓
Add $NaHPO_4$ to red blood cells
↓
Lyse red blood cells
↓
0.1-μm filtration
↓
500-kDa ultrafiltration
↓
10-kDa ultrafiltration/concentration
↓
Deoxygenate hemoglobin
↓
Cross-link hemoglobin with bis(3,5-dibromosalicyl)fumarate (DBBF)
↓
Adjust to pH 9.0 with HEPES
↓
0.1-μm filtration
↓
500-kDa ultrafiltration
↓
10-kDa ultrafiltration/concentration
↓
Buffer exchange to pH 7.4 with Ringer's acetate
↓
Sterile fill cross-linked stroma-free hemoglobin (DBBF-SFH)

FIG. 6. Flow diagram for production of stroma-free hemoglobin; this production scheme is a simplification of that utilized by the Blood Research Division, Letterman Army Institute of Research, The Presidio, San Francisco, California.

endotoxin-free) conditions. An example of a midprocess contamination that was detected by LAL testing of samples at multiple stages is shown (Fig. 7). Because the site responsible for the endotoxin contamination was clearly identified, reestablishment of an endotoxin-free state at the specific step could be approached and accomplished efficiently. Although

SAMPLE	LPS (pg/ml)*
Water rinse of tank	<1
Water rinse of 0.45-μm membrane	<1
NaCl from wash tank	<1
Red blood cells from wash tank	<1
Concentrated red blood cells	<1
Sodium phosphate buffer	<1
Water rinse of 0.1-μm membrane	<1
Water rinse of 10-kDa membrane	<1
Water rinse of concentration tank	<1
Hemoglobin postred blood cell lysis	<1
0.1-μm membrane permeate SFH	10
0.1-μm membrane retentate (stroma)	100**
500-kDa membrane retentate	10
500-kDa membrane filtrate SFH	10
Concentrated SFH	100**
Bagged DBBF-SFH final product	300**

FIG. 7. LAL testing of the production facility and selected steps during the production of an endotoxin-contaminated solution of cross-linked stroma-free hemoglobin (DBBF–SFH). *, Based on an *E. coli* B 055:B5 standard; **, note that relatively high concentrations of endotoxin were detected in the 0.1-μm membrane retentate, in a sample of concentrated SFH, and in the final bagged product. This indicates that breaks in the endotoxin-free technique occurred during the removal of stroma and during the final concentration step.

endotoxin contamination of hemoglobin frequently follows the addition of reagents or sample manipulations (e.g., stabilization of hemoglobin by chemical cross-linking with DBBF or other cross-linking agents), we also have observed that the erythrocytes that constitute the starting material may be contaminated with endotoxin, with subsequent contamination of the purified hemoglobin. Prevention of endotoxin contamination of hemoglobin is far more efficient and economical than attempted removal of endotoxin from the final protein product; therefore, in the following section, we describe several basic procedures to minimize the likelihood of endotoxin contamination.

Depyrogenation Techniques

It is important to recognize that endotoxin has a strong affinity for a wide variety of surfaces and that removal of endotoxin from equipment

used for the production of any intravenous product can be technically difficult. Washing of the production apparatus with endotoxin-free solutions (such as those prepared for intravenous use) is often effective for the removal of endotoxin, although extremely large volumes of wash solutions may be required to render systems endotoxin free.** In the case of chromatographic columns, for example, 10–20 or more column volumes of wash solution may be required to generate a LAL-negative system. To make possible the production of endotoxin-free hemoglobin, all glassware and metal containers must be heated at high temperature (180–200° for 3–4 hr) to inactivate endotoxin. Solutions and buffers may be filtered through 10–100 kDa membranes to remove endotoxin, and the erythrocytes used as the starting source of hemoglobin should be LAL tested to establish that no contamination had occurred during procurement of the blood. Sterility is not adequate to ensure lack of endotoxin, because wet, 120° heat is not sufficient to denature endotoxin. Thus, any plasticware used during production of hemoglobin must be LAL tested. In-line filters and membranes can also be a source of endotoxin contamination, and therefore wash solutions should be passed through these sites and subsequently LAL tested prior to use of the apparatus for generation of hemoglobin. When a source of endotoxin contamination is detected, it may be possible to remove endotoxin by means of chemical denaturation. A variety of such treatments have been described (Table II), although the efficacy of these methods has not been reported for hemoglobin production. The use of lipopolysaccharide-binding substances (e.g., polymyxin B or an endotoxin-neutralizing protein), and potentially of other proteins from a recognized family of endotoxin-binding proteins,[23] may provide the basis for removal of endotoxin from solutions when these LPS-binding proteins are immobilized and used to remove endotoxin by affinity binding.

Product quality control requirements of the FDA for intravenous preparations include documentation by LAL testing of adequately low endotoxin concentrations. The acceptable endotoxin concentration threshold is defined according to endotoxin units (EU) per milliliter, because the biological activities, both in the *Limulus* test and *in vivo*, of various endotoxins are widely variable on a weight basis.[24–27] Commercial LAL re-

** Because pharmaceutical companies no longer routinely utilize the rabbit pyrogen test, it is technically inappropriate to use the term "pyrogen free."

[23] P. S. Tobias, J. C. Mathison, and R. J. Ulevitch, *J. Biol. Chem.* **263,** 13479 (1988).

[24] M. E. Weary, G. Donahue, F. C. Pearson, and K. Story, Appl. Environ. Microbiol. **40,** 1148 (1980).

[25] M. E. Weary, F. C. Pearson, J. Bohon, and G. Donahue, *in* "Endotoxins and their Detection with the *Limulus* Amebocyte Lysate Test" (S. W. Watson, J. Levin, and T. J. Novitsky, eds.), p. 365. Alan R. Liss, New York, 1982.

TABLE II
TECHNIQUES FOR DEPYROGENATION OF SOLUTIONS

Technique	Ref.
Ultrafiltration	*a, b*
Polymyxin B	*c, d*
Endotoxin-neutralizing protein	*e*
Acid hydrolysis	*f*
Basic hydrolysis	*g*
Oxidation by hydrogen peroxide	*h*
Alkylation with succinic anhydride	*i*
Barium sulfate adsorption	*j*
Endotoxin–protein dissociation with octyl-β-D-glucopyranoside	*k*
Ultracentrifugation	*l*
Endotoxin removal by LAL gelation	*m*

[a] K. J. Sweadner, M. Forte, and L. L. Nelson, *Appl. Environ. Microbiol.* **34,** 382 (1977).

[b] L. W. Henderson and E. Beans, *Kidney Int.* **14,** 522 (1978).

[c] D. C. Morrison and D. M. Jacobs, *Immunochemistry* **13,** 813 (1976).

[d] D. M. Jacobs and D. C. Morrison, *J. Immunol.* **118,** 21 (1977).

[e] N. R. Wainwright, R. J. Miller, E. Paus, T. J. Novitsky, M. A. Fletcher, T. M. McKenna, and T. Williams, *in* "Cellular and Molecular Aspects of Endotoxin Reactions" (A. Nowotny, J. J. Spitzer, and E. J. Ziegler, eds.), p. 315. Elsevier, Amsterdam, 1990.

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agents are available that demonstrate sufficient sensitivity and reproducibility to ensure that hemoglobin products satisfy the FDA requirements. Parenteral formulations such as hemoglobin for resuscitation have an allowable endotoxin concentration (termed the endotoxin limit) that varies according to the volume of infusate and weight of the recipient: endotoxin limit = 5.0 EU/kg/maximal human dose (as prescribed by the FDA).[28]

Technical Issues Related to LAL Testing of Hemoglobin

The following technical considerations concerning endotoxin testing of hemoglobin preparations are important. First, different endotoxins may demonstrate significant differences both in their *in vivo* physiological effects and *in vitro* biological activities. Environmental endotoxins that can cause contamination of hemoglobin are variable in regard to bacterial species, purity (e.g., extent of association of bacterial lipid, protein, and nucleic acids with lipopolysaccharide), and relative biological potencies, as determined by the LAL test, and are not necessarily similar to a standard endotoxin preparation.[26,27]

Second, there are uncertainties in the derivation of the concentration of an unknown endotoxin from a standard endotoxin curve. Standard reference endotoxins (e.g., EC-5) are much more purified than are environmental endotoxins, and their LAL reactivity on a weight basis may be significantly different from that of environmental endotoxins. Nevertheless, for purposes of quality control, we recommend the use of stable, available standard endotoxins such as EC-5 or *E. coli* B 055:B5, which are not totally purified. These endotoxin preparations are structurally and chemically more similar to "native" environmental endotoxins than are synthetic endotoxins, endotoxin partial structures, or lipid A. They are also similar to the gut-derived endotoxins with which infused hemoglobin solutions are likely to interact in patients with major trauma and bacteremia. If necessary, the activity of the endotoxin selected as a standard can be related to the activity of EC-5, the current FDA standard endotoxin.

Third, detection of endotoxin in protein solutions with the LAL test (or other tests for endotoxin) may be complicated by the phenomena of enhancement and inhibition.[29] In studies of cross-linked stroma-free

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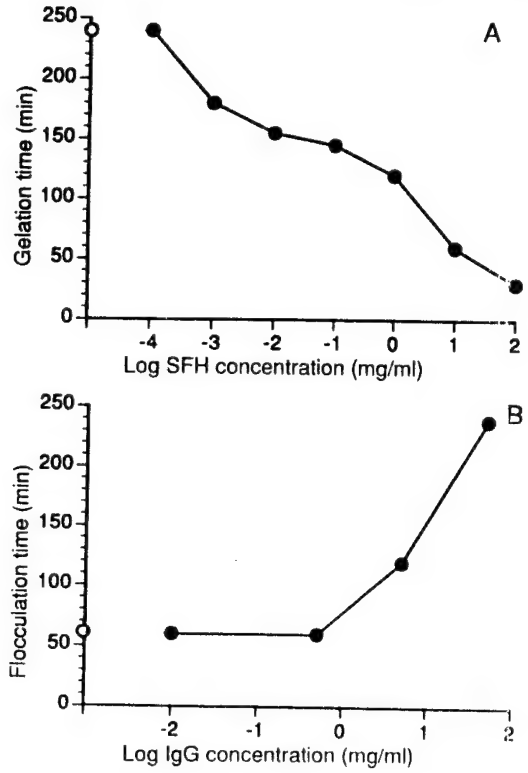

FIG. 8. (A) Enhancement of *Limulus* lysate reactivity for bacterial endotoxin by stroma-free hemoglobin (SFH). The 0.05-ml *Limulus* lysate sample was incubated with 0.05 ml of *E. coli* endotoxin (100 ng/ml) at 37°, in the presence of various concentrations (●) of endotoxin-free stroma-free hemoglobin (100 ng/ml–100 mg/ml). In the absence of added hemoglobin (○), gelation of *Limulus* lysate was observed in 240 min. Gelation time of *Limulus* lysate was decreased by the addition of hemoglobin in a concentration-dependent manner. This results in an apparent increase in the concentration of endotoxin. (B) Inhibition of *Limulus* lysate reactivity for bacterial endotoxin by IgG. The 0.05-ml *Limulus* lysate sample was incubated with 0.05 ml of *E. coli* endotoxin (100 pg/ml) at 37°, in the presence of various concentrations of endotoxin-free IgG (10 μg/ml–50 mg/ml) (●). In the absence of IgG (○) or in the presence of 0.01 or 0.5 mg/ml IgG, flocculation of *Limulus* lysate was observed after 60 min, with gelation at 3 hr. In the presence of 5 or 50 mg/ml IgG, *Limulus* lysate did not form a gel, and flocculation time was increased in a concentration-dependent manner.

hemoglobin, enhanced detection of spiked endotoxin was shown to be directly related to the concentration of hemoglobin (Fig. 8A). This potential enhancement effect, which was not detected in a previous report,[30] should be taken into account. It may be necessary to determine whether

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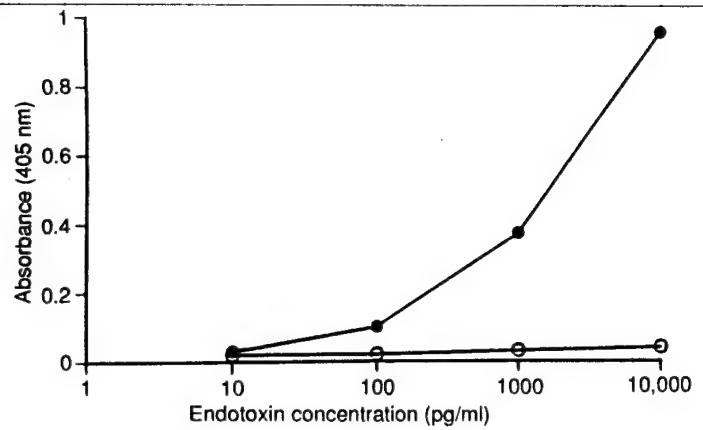

FIG. 9. Detection of bacterial endotoxin in plasma following inhibitor inactivation. Human plasma was spiked with *E. coli* endotoxin (final concentrations from 10 pg/ml to 10 ng/ml), and aliquots then were diluted 10-fold in endotoxin-free 0.15 *M* NaCl and heated at 65° for 30 min. Other aliquots were untreated. The 0.05-ml plasma samples subsequently were added to 0.05 ml of *Limulus* lysate, incubated at 37° for 30 min, and then chromogenic substrate S-2222 was added. Absorbance of released pNA was measured at 405 nm. Spiked endotoxin was not detected in untreated plasma (○), whereas dilution and heating of plasma (●) resulted in concentration-dependent detection of bacterial endotoxin.

the apparent concentration of endotoxin, which may have been elevated secondary to enhancement, is physiologically relevant when evaluated by other *in vivo* endotoxin assays or by the rabbit pyrogen test, and eventually by administration to humans. Inhibition of detection of endotoxin in the LAL test also is a well-recognized problem with some protein solutions for intravenous use, as illustrated in Fig. 8B for γ-globulin. In the presence of γ-globulin, inhibition of detection of spiked endotoxin was shown to be directly related to IgG concentration. Inhibition also has been shown to interfere with the detection and/or quantification of endotoxin in blood, and, therefore, *in vivo* studies of endotoxemia and hemoglobin infusion cannot be accomplished without treatment of blood to allow the detection of LPS.[31] Endotoxemia can be recognized only after neutralization of plasma inhibitors. LAL detection of LPS in plasma is best achieved using a dilution/heating method for inhibitor neutralization, as shown in Fig. 9. Other plasma treatment methods, e.g., chloroform extraction and acid oxidative techniques, have been evaluated but are not as successful at recovery and detection of endotoxin.[31] Inhibition of detection of endotoxin

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by plasma proteins is also a well-recognized problem in other assays for bacterial endotoxins.[32]

Acknowledgments

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TOXICITY OF HEMOGLOBIN SOLUTIONS: HEMOGLOBIN IS A LIPOPOLYSACCHARIDE (LPS) BINDING PROTEIN WHICH ENHANCES LPS BIOLOGICAL ACTIVITY

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ABSTRACT

Administration of αα-crosslinked stroma-free hemoglobin (SFH) as a cell-free resuscitation fluid is associated with multiple organ toxicities. Many of these toxicities are characteristic of the pathophysiological effects of bacterial endotoxins (lipopolysaccharide, LPS). To better understand the potential role of LPS in the observed in vivo toxicities of SFH, we examined mixtures of SFH and E. coli LPS for evidence of LPS-SFH complex formation. LPS-SFH complexes were demonstrated by three techniques: ultrafiltration through 300 kDa cut-off membranes, which distinguished LPS in complexes (87-89% <300 kDa) from LPS alone (90% >300 kDa); density centrifugation through 5% sucrose, which distinguished denser LPS alone from LPS-SFH complexes; and precipitation by 67% ethanol, which demonstrated 2-3 fold increased precipitability of complexes compared to SFH alone. Interaction of LPS with SFH was also associated with markedly increased biological activity of LPS, as manifested by enhancement of

LPS activation of Limulus amebocyte lysate (LAL), increased release of human mononuclear cell tissue factor, and enhanced production of cultured human endothelial cell tissue factor. These results demonstrated that hemoglobin can serve as an endotoxin binding protein, and that this interaction results in the alteration of several LPS physical characteristics and enhancement of LPS biological activities.

INTRODUCTION

Stroma-free hemoglobin, a preparation of purified human hemoglobin, is being developed for use as a cell-free resuscitation fluid [1-3]. In order to stabilize the protein's tetrameric structure, preparations of stroma-free hemoglobin have been covalently crosslinked between the protein chains. αα-crosslinked stroma-free hemoglobin (SFH) is a modified hemoglobin, crosslinked between the α chains with bis(3,5-dibromosalicyl) fumarate, that demonstrates prolonged in vivo retention. SFH has excellent oxygen binding and delivery properties, as well as an adequate half-life, and therefore is a potentially ideal "blood substitute". However, in vivo administration of SFH has revealed significant problems of toxicity, including hypertension and bradycardia [4,5], a decrease in glomerular filtration rate and renal plasma flow [6], mild prolongations of the partial thromboplastin time [5] and fever. In some studies, administration of SFH has resulted in activation of the complement and coagulation cascades [7-9], disseminated intravascular coagulation with resultant thrombosis [7,10,11], and ischemic parenchymal damage [7,8].

Many of the reported toxicities of SFH infusion can be explained by the known consequences of endotoxemia, and the presence of LPS in preparations of SFH utilized for in vivo studies has been documented [7,9]. Therefore, a contributory role for the observed in vivo toxicity of SFH has been proposed for bacterial endotoxin [10,11]. Previously, SFH and LPS have been shown to produce synergistic in vivo toxicity [11], and we have demonstrated that SFH is capable of enhancing the procoagulant activity of LPS in vitro [12]. We hypothesized that SFH

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We hypothesized that SFH

binds LPS, and that the interaction between these molecules could alter the biological activity of LPS. The present study was designed to determine whether complex formation occurs between SFH and LPS, and evaluate the ability of SFH to alter biologic activities of LPS.

MATERIALS AND METHODS

Reagents. Sterile, 15 ml Falcon tubes were obtained from Becton Dickinson (Mountainview, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL).

Glassware. Glassware was heated at 190°C in a dry oven for 4 hours.

Hemoglobin. Human SFH, crosslinked between α chains with bis(3,5-dibromosalicyl)fumarate as described previously [13,14], was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA. SFH was 9.6 g/dl, pH 7.4, 95.4% crosslinked, 96.3% oxyhemoglobin, 3.2% methemoglobin, and contained less than 0.4 EU/ml endotoxin (referenced to E. coli lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test [15]. The SFH stock solution was stored at -70°C , and then diluted with sterile, pyrogen-free 0.9% NaCl prior to use. Carboxy-SFH(CO-SFH), produced by incubation of the SFH solution with CO, was at 9.6 g/dl, 95.4% crosslinked, 95% HbCO and 5% oxyhemoglobin. Purified non-crosslinked human A_0, 8.4 g/dl, also provided by collaborators at BRD/LAIR, was prepared by ion exchange HPLC of purified human hemoglobin, as described previously [16].

Endotoxin. E. coli O26:B6 lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). ^{14}C-LPS (Salmonella typhimurium PR122(Rc)) was purchased from List Biologicals, Inc. (Campbell, CA) and was resuspended in endotoxin-free water at 1 μCi/ml (1 mg/ml).

Limulus amebocyte lysate (LAL). Amebocyte lysates were prepared from Limulus polyphemus (the North American horseshoe crab) by disruption of washed amebocytes in distilled water [15,17].

Chromogenic Substrate. Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger.

Chromogenic Limulus Amebocyte Lysate (LAL) Test. 50 µl of sample and 30 µl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were incubated in tissue culture plates for 30 min at 37°C in a temperature-controlled plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). 40 µl chromogenic substrate S-2423 (0.25 mM, in 25 mM Tris, pH 8.6) was added to each well, mixtures were incubated at 37°C for 5 min, and absorbances at 405 nm then were determined.

Ultrafiltration. Solutions of SFH, CO-SFH or A_0 were prefiltered through an endotoxin-free 300 kDa membrane prior to use to remove aggregated protein particles. SFH, CO-SFH or A_0 (100 µg/ml) was incubated with E. coli O26:B6 (W) LPS (50 µg/ml) for 30 min at 37°C. Mixtures then were filtered manually with a 3 ml syringe (according to the directions of the filter manufacturer) at room temperature, using a 300 kDa cut-off filter (ultrafree-PFL polysulfone 300, Millipore Corporation, Bedford, MA). LPS concentrations in filtered solutions of hemoglobin, hemoglobin and LPS mixtures, or LPS alone were determined by the chromogenic LAL test (described above), using starting mixtures of hemoglobin-LPS, or LPS alone, for the standard curve. Hemoglobin protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

Sucrose centrifugation of LPS and SFH. ^{14}C-S. typhimurium LPS (0.005 µCi) was added to each of the hemoglobin solutions (each diluted to 10 mg/ml), and the mixtures were incubated for 30 min at 20°C. Aliquots of LPS-hemoglobin mixtures, LPS alone, or hemoglobin alone then were layered over 5% pyrogen-free sucrose and centrifuged at 2,900 x g for 30 min at 20°C, in a Sorvall RC-5 centrifuge (Du Pont Instruments, Wilmington, DE). Scintillation counting was performed, after samples were diluted 10-fold in fluor (Formula A-989, NEN Research Products, Boston, MA), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). For samples containing hemoglobin, quenching of ^{14}C-LPS by hemoglobin was reversed as follows: 0.1 ml aliquots of fractions were diluted ten-fold in water (to 1 ml final volume), and 1 ml Solvable (NEN Research Products, Boston, MA) was added. These mixtures were incubated at 60°C for one hr, and then 0.3 ml 25% H_2O_2 was added.

Ethanol precipitation of SFH
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Ethanol precipitation of SFH and LPS-SFH mixtures. 2 µg SFH, CO-SFH, or A_0 was incubated with 25 µg E. coli O26:B6 (W) LPS in microtiter plate wells for 30 min at 20°C. Ethanol then was added to each well (final concentration, 67%), and after an additional 30 min the mixtures were centrifuged at 800 x g for 30 min. The concentrations of hemoglobin in the sediments were determined by protein assay, and LPS concentrations by the phenol-concentrated H_2SO_4 method [18].

Mononuclear cell (MNC) tissue factor (TF) assay. E. coli LPS (100 ng/ml) was incubated with SFH (6 mg/ml) for 30 min at 37°C. LPS alone, SFH alone, or LPS-SFH mixtures were then incubated for 20 hr at 37°C with human peripheral blood MNC [19] and assayed for TF with a one-stage coagulation assay [20]. A clotting time of 30 sec was defined as equal to 100 units TF activity [21].

Endothelial cell tissue factor (TF) assay. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured in media (containing 2% serum) obtained from Clonetics. Cells were grown to confluent monolayers in sterile 96-well microtiter plate wells (Nunclon, Applied Scientific, San Francisco). E. coli LPS alone, SFH alone, or LPS-SFH mixtures were added to the media in each well (final concentrations: 1 µg/ml LPS; 1 mg/ml SFH), and incubated for 4 hr. Wells were then washed with media (x 3) and the HUVEC were freeze-thawed (x 2) and sonicated in phosphate buffered saline. To each well then was added normal human citrated plasma and calcium (25 mM), and plates were incubated for 8 min in a temperature-controlled (37°C) plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). Turbidity was measured at 340 nm, and TF activity was calculated from a standard curve established with rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 min by 1:100 diluted thromboplastin was arbitrarily defined as 10 TF units.

RESULTS

Demonstration of LPS-SFH complexes. Ultrafiltration experiments demonstrated that 87-89% of the LPS in LPS-SFH mixtures was

filtered through the 300 kDa membrane, whereas only 10% of LPS alone was filterable (Fig. 1). This indicated that SFH caused the dissociation of LPS into lower molecular weight particles. Approximately 90% of the total SFH protein in each of the three LPS-SFH mixtures, and from filtrates of SFH alone, was detected in filtrates (data not shown). Utilizing ethanol precipitation, greater than twice the amount of each SFH was precipitated in the presence of LPS than was with SFH alone (Fig. 2). In both the absence and presence of SFH, approximately 90% of LPS was precipitated by ethanol (data not shown). Following sucrose centrifugation, 76% of LPS sedimented into the bottom fraction in the absence of protein, whereas only 3-9% sedimented in the presence of any of the three SFH preparations (Figure 3). Conversely, only 3% of LPS alone remained above the sucrose layer, whereas in the presence of SFH, 64-79% of LPS remained in the top layer. No detectable SFH entered the sucrose layer in either the absence or presence of LPS. Therefore, SFH decreased the density of LPS, resulting in the co-migration of SFH and LPS.

Biological activity of LPS in SFH-LPS complexes. SFH increased the biological activity of LPS in three independent assays. First, LPS in the presence of SFH produced enhanced activation of LAL (3 to 4.5-fold) compared to LAL activation by LPS alone (Fig. 4). Second, LPS-SFH complexes resulted in 5.5-fold greater TF production by human MNC than the TF generated from MNC by LPS alone (Fig. 5). Third, SFH resulted in a 2.8-fold increase in endothelial cell TF production compared to TF generated by LPS alone (Fig. 6).

DISCUSSION

We performed experiments to determine whether SEH interacted with LPS. Ultrafiltration demonstrated that the molecular weight of LPS (typically $>10^6$ in aqueous solution) was reduced to < 300 kDa in the presence of SFH, and that LPS and SFH co-filtered. Utilizing centrifugation through sucrose, we showed that the density of LPS in the presence of SFH was distinctly less than that of LPS alone, and that LPS and SFH co-migrated. Measurement of SFH precipitation by

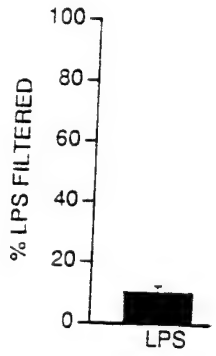

FIGURE 1. Ultrafiltration with SFH, CO-SFH or A_0 300 kDa cut-off ultrafiltra deter-mined by the LAL te: creased the filterability of I

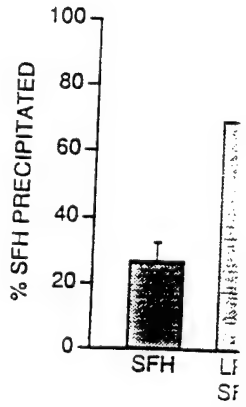

FIGURE 2. Precipitation of incubated with SFH, CO-S were then precipitat-ed fr sedimented by cen-trifugatio increased precipitability in t.

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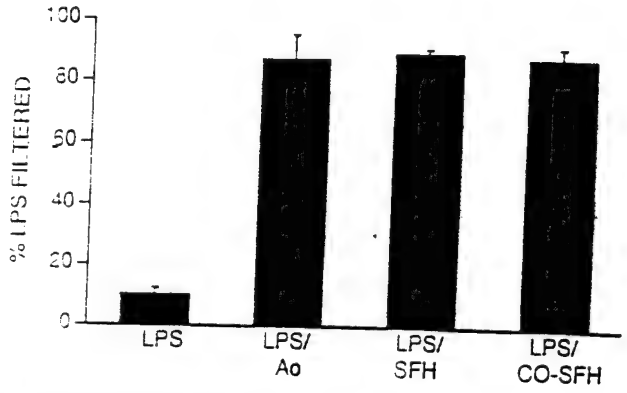


FIGURE 1. Ultrafiltration of SFH and LPS. E. coli LPS was incubated with SFH, CO-SFH or A_o, and the mixtures were filtered through a 300 kDa cut-off ultrafiltration membrane. The % of LPS filtered was deter-mined by the LAL test. All three preparations of SFH greatly increased the filterability of LPS.

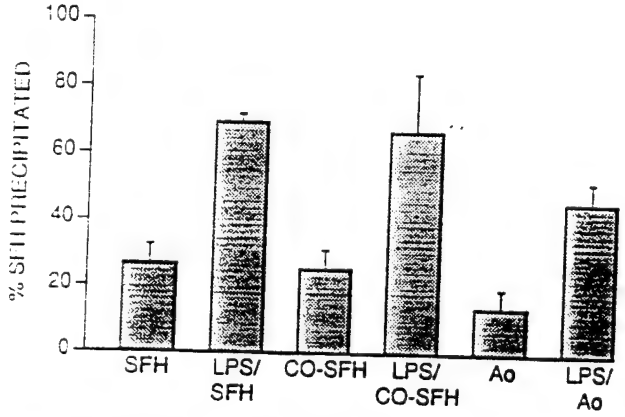


FIGURE 2. Precipitation of SFH and LPS by ethanol. E. coli LPS was incubated with SFH, CO-SFH, or A_o, and the LPS-SFH complexes were then precipitat-ed from the mixtures by 67% ethanol and sedimented by cen-trifugation. Each preparation of SFH demonstrated increased precipitability in the presence of LPS.

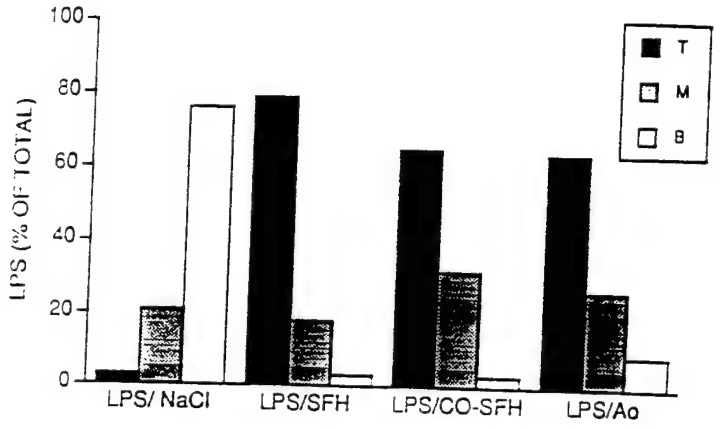


FIGURE 3. Centrifugation of SFH and LPS through sucrose. ^{14}C S. typhimurium LPS was incubated with SFH, CO-SFH, or A_o, and the mixtures were then centrifuged through 5% sucrose. The distributions of radiolabeled LPS were determined in top (T), middle (M) and bottom (B) zones of the centrifuged samples. All three preparations of SFH co-migrated with LPS, resulting in a decrease in LPS density.

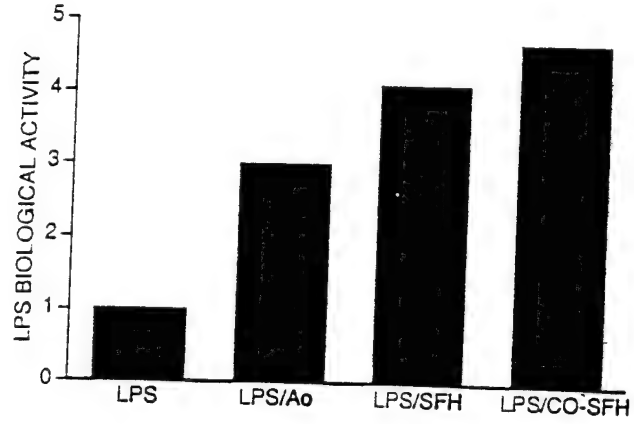


FIGURE 4. Effect of SFH on LPS biological activity in the LAL test. E. coli LPS, in the absence or presence of SFH, CO-SFH, or A_o was incubated with LAL, and activation measured with a chromogenic substrate. LPS biological activities in LPS-protein mixtures are expressed as relative activities to LPS alone. All three preparations of SFH resulted in increased biological activity of LPS.

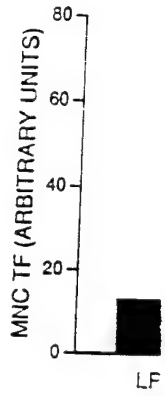


FIGURE 5. Effect of activity in human MN SFH, was incubated wit with a plasma clotting stimulate TF production

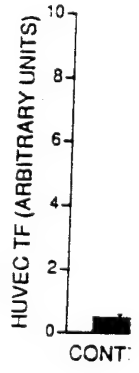
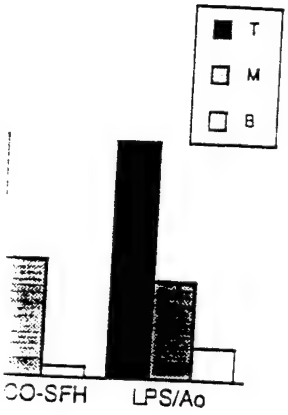


FIGURE 6. Effect of S activity in HUVEC. E. was incubated with HU plasma clotting assay. S TF from the endothelial c



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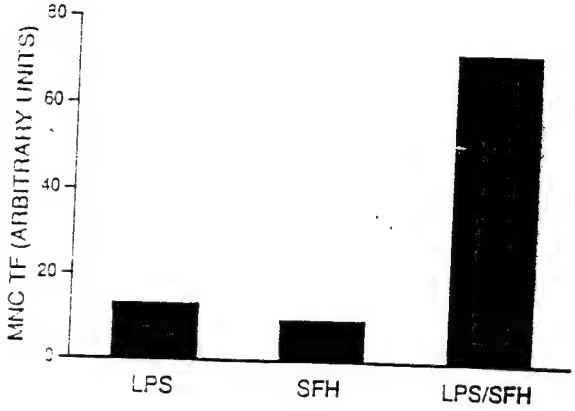

FIGURE 5. Effect of SFH on the LPS-induced stimulation of TF activity in human MNC. E. coli LPS, in the absence or presence of SFH, was incubated with human MNC, and TF activity was measured with a plasma clotting assay. SFH enhanced the ability of LPS to stimulate TF production.

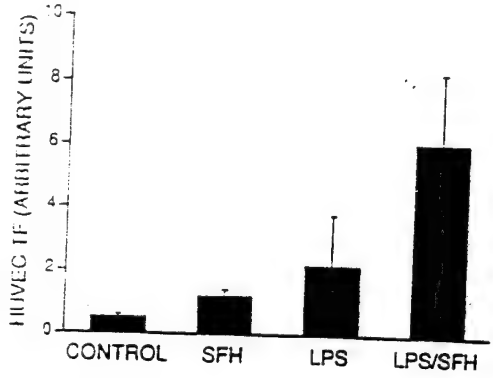

FIGURE 6. Effect of SFH on the LPS-induced stimulation of TF activity in HUVEC. E. coli LPS, in the absence or presence of SFH, was incubated with HUVEC, and TF activity was measured with a plasma clotting assay. SFH enhanced the LPS-induced production of TF from the endothelial cells.

ethanol indicated that LPS greatly increased the precipitability of SFH. Therefore, our experiments demonstrated that the physical characteristics of both SFH and LPS were altered in LPS-SFH mixtures. These results are consistent with the formation of stable complexes, and establish the ability of hemoglobin to act as an endotoxin-binding protein. Because these results were observed with unmodified hemoglobin (A_0) and CO-SFH (which was not susceptible to methemoglobin production), as well as with SFH, we have demonstrated that LPS-binding is an intrinsic property of hemoglobin.

The formation of LPS-SFH complexes was associated with major changes in the procoagulant activities of LPS. SFH enhanced the ability of LPS to stimulate coagulation via three independent mechanisms: 1) direct activation of the proteolytic coagulation cascade of Limulus, 2) stimulation of TF production from human MNC, and 3) stimulation of TF production from HUVEC. Enhancement by SFH of LPS procoagulant activity may contribute to the observed thrombosis and ischemic damage associated with SFH infusion in animals [7,8], and may also provide a mechanism for the synergistic toxicity between SFH and LPS reported previously [11,22]. Interestingly, other proteins that are known to bind LPS with a resultant change in LPS biological activity (e.g., mellitin [23], lysozyme [24], and complement [25] or the polypeptide polymyxin B [26]) cause a decrease in LPS toxicity.

Our observations that LPS, when complexed with SFH, was of much lower molecular weight and lesser density than LPS alone suggest that SFH caused the disaggregation of LPS. In contrast to the increased biological activity we observed for LPS that had been disaggregated and bound to SFH, the process of LPS disaggregation in plasma (resulting primarily from its interaction with high density lipoproteins [27]), results in detoxification. It is possible that the process of LPS-SFH complex formation might potentially interfere with LPS detoxification in plasma.

Acknowledgments: This work was supported, in part, by the U.S. Army Medical Research and Development Command Research Contract Log Number 88270001/MIPR No. 90 MM0535. Opinions, interpretations,

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We particularly thank Dr. Jack Levin for helpful discussions of experimental design and interpretation of results. Francine C. Levin provided excellent technical assistance. Determination of tissue factor levels in mononuclear cells was performed in the laboratory of Dr. Frederick Rickles, University of Connecticut School of Medicine, Farmington, CT.

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HEMOGLOBIN: A NEWLY RECOGNIZED BINDING PROTEIN FOR BACTERIAL ENDOTOXINS (LPS)

Robert I. Roth, Wieslaw Kaca and Jack Levin

From the Department of Laboratory Medicine, University of California School of Medicine, and the Veterans Administration Medical Center, San Francisco, CA 94121

Abbreviations: Hemoglobin (Hb), crosslinked cell-free hemoglobin (ααHb), lipopolysaccharide (LPS), Limulus amebocyte lysate (LAL), tissue factor (TF), mononuclear cell (MNC), human umbilical vein endothelial cells (HUVEC)

ABSTRACT

Administration of purified hemoglobin (Hb) as a cell-free resuscitation fluid is associated with multiple organ toxicities. Many of these toxicities are characteristic of the pathophysiological effects of bacterial endotoxins (lipopolysaccharide, LPS). To better understand the potential role of LPS in the observed in vivo toxicities of Hb, we examined mixtures of Hb and LPS for evidence of LPS-Hb complex formation. LPS-Hb complexes were demonstrated by three techniques: ultrafiltration through 300 kDa cut-off membranes, which distinguished LPS in complexes (87-89% <300 kDa) from LPS alone (90% >300 kDa); density centrifugation through sucrose, which distinguished denser LPS alone from LPS-Hb complexes; and precipitation by 67% ethanol, which demonstrated 2-3 fold increased precipitability of Hb in complexes compared to Hb alone. Interaction of LPS with Hb was also associated with markedly increased biological activity of LPS, as manifested by enhancement of LPS activation of Limulus amebocyte lysate (LAL), increased release of human mononuclear cell tissue factor, and enhanced production of human endothelial cell tissue factor. These results demonstrated that hemoglobin can serve as an endotoxin binding protein, and that this interaction results in the alteration of several of the physical characteristics of LPS and enhancement of the biological activities of LPS. These findings suggest that a mechanism for the toxicity of infused Hb in vivo may involve potentiation of the biological effects of LPS. In addition, these observations suggest a mechanism by which LPS-related morbidity during sepsis could be enhanced by erythrocyte hemolysis.

INTRODUCTION

Several studies have been conducted using cell-free preparations of purified human hemoglobin (Hb) that were developed for use as a cell-free resuscitation fluid (DeVenuto, Zegna, 1982; Sehgal et al., 1984; Winslow, 1989). Preliminary

experiments in our laboratory have suggested that human hemoglobin may have a physiologically important interaction with endotoxin (lipopolysaccharide, LPS). Previously, an association between LPS and Hb had been suggested by the observations that in vivo administration of Hb produced the following toxicities: activation of the complement and coagulation cascades (Feola, et al., 1988a; Feola, et al., 1988b; Marks, et al., 1989), disseminated intravascular coagulation with resultant thrombosis (Feola, et al., 1988a; White, et al., 1986a; White, et al., 1986b), ischemic parenchymal damage (Feola, et al., 1988a; Marks, et al., 1989), hypertension and bradycardia (Amberson, et al., 1949; Savitsky, et al., 1978), a decrease in glomerular filtration rate and renal plasma flow (Brandt, et al., 1951), and mild prolongations of the partial thromboplastin time (Savitsky, et al., 1978). Since these toxicities can in large part be explained by the known consequences of endotoxemia, and since the presence of LPS in preparations of Hb utilized for in vivo studies has been documented (Feola, et al., 1988a; Marks, et al., 1989), it has been proposed that LPS has a contributory role in the observed in vivo toxicity of Hb infusions (White, et al., 1986a; White, et al., 1986b). Previously, Hb and LPS have been shown to produce synergistic in vivo toxicity (White, et al., 1986b), and we have demonstrated that Hb is capable of enhancing the procoagulant activity of LPS in vitro (Roth, Levin, 1994). Therefore, we hypothesized that Hb binds LPS, and that the interaction between these molecules could alter the biological activity of LPS. The present study was designed to determine whether complex formation occurs between Hb and LPS, and evaluate the ability of Hb to alter biologic activities of LPS.

MATERIALS AND METHODS

Reagents. Sterile, 15 ml Falcon tubes were obtained from Becton Dickinson (Mountain-view, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL).

Glassware. Glassware was heated at 190°C in a dry oven for 4 hours.

Hemoglobin. Human Hb, covalently crosslinked between α chains (ααHb) with bis(3,5-dibromo-salicyl) fumarate as described previously (Winslow, et al., 1991; Chatterjee, et al., 1986) in order to stabilize the protein's tetrameric structure, and purified non-crosslinked human A_0 (HbA_0) prepared by ion exchange HPLC of purified human Hb as described previously (Christensen, et al., 1988), were provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA. Hb preparations contained less than 0.4 EU/ml endotoxin (referenced to E. coli lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate (LAL) test (Levin, Bang, 1968).

Endotoxin. E. coli O26:B6 lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). ^{14}C-LPS (Salmonella typhimurium PR122(Rc)) was purchased from List Biologicals, Inc. (Campbell, CA) and was resuspended in endotoxin-free water at 1 μCi/ml (1 mg/ml). Proteus mirabilis LPSs (smooth strains S1959 and 03; rough mutant R110; and deep rough mutant R45 LPS) were provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland.

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Limulus amebocyte lysate (LAL). Amebocyte lysates were prepared from Limulus polyphemus (the North American horseshoe crab) by disruption of washed amebocytes in distilled water (Levin, Bang, 1964; Levin, Bang, 1968).

Chromogenic substrate. Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger.

Chromogenic Limulus amebocyte lysate (LAL) test. 50 µl of sample and 30 µl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were incubated in tissue culture plates for 30 min at 37°C in a temperature-controlled plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). 40 µl chromogenic substrate S-2423 (0.25 mM, in 25 mM Tris, pH 8.6) was added to each well, mixtures were incubated at 37°C for 5 min, and absorbances at 405 nm then were determined.

Ultrafiltration. Solutions of Hb were prefiltered through an endotoxin-free 300 kDa membrane prior to use to remove aggregated protein particles. Hb (100 µg/ml) was incubated with E. coli O26:B6 LPS (50 µg/ml) for 30 min at 37°C. Mixtures then were filtered manually with a 3 ml syringe (according to the directions of the filter manufacturer) at room temperature, using a 300 kDa cut-off filter (ultrafree-PFL polysulfone 300, Millipore Corporation, Bedford, MA). LPS concentrations in filtered solutions of Hb, Hb and LPS mixtures, or LPS alone were determined by the chromogenic LAL test (described above), using starting mixtures of Hb-LPS, or LPS alone, for the standard curve. Hb protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

Sucrose centrifugation of LPS and Hb. ^{14}C-S. typhimurium LPS (0.005 µCi) was added to each of the Hb solutions (each diluted to 10 mg/ml), and the mixtures were incubated for 30 min at 20°C. Aliquots of LPS-Hb mixtures, LPS alone, or Hb alone then were layered over 5% pyrogen-free sucrose and centrifuged at 2,900 x g for 30 min at 20°C, in a Sorvall RC-5 centrifuge (Du Pont Instruments, Wilmington, DE). Scintillation counting was performed, after samples were diluted 10-fold in fluor (Formula A-989, NEN Research Products, Boston, MA), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). For samples containing Hb , quenching of ^{14}C-LPS by Hb was reversed as follows: 0.1 ml aliquots of fractions were diluted ten-fold in water (to 1 ml final volume), and 1 ml Solvable (NEN Research Products, Boston, MA) was added. These mixtures were incubated at 60°C for one hr, and then 0.3 ml 25% H_2O_2 was added.

In other experiments, aliquots of LPS-Hb mixtures, LPS alone, or Hb alone were layered over a sucrose gradient (4-20%) and centrifuged at 52,000 x g for 4 hr at 20°C, in a Sorvall RC70 centrifuge and T641 swinging bucket rotor (Du Pont Instruments, Wilmington, DE). After centrifugation, fractions through the gradient were obtained and analyzed for ^{14}C-LPS and Hb, as described above.

Ethanol precipitation of Hb and LPS-Hb mixtures. Hb (2 µg) was incubated with E. coli O26:B6 LPS (25 µg) in microtiter plate wells for 30 min at 4°C, 20°C or 37°C. Ethanol then was added to each well (final concentration, 67%), and after an additional 30 min the

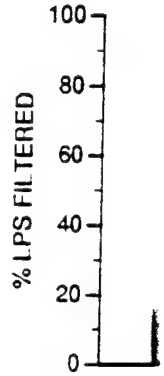
mixtures were centrifuged at 800 x g for 30 min. The concentrations of Hb in the sediments were determined by protein assay, and LPS concentrations by the phenol-concentrated H_2SO_4 method (Nowotny, 1979).

Mononuclear cell (MNC) tissue factor (TF) assay. E. coli LPS (100 ng/ml) was incubated with Hb (range 0.6-60 mg/ml) for 30 min at 37°C. LPS alone or LPS-Hb mixtures were then incubated for 20 hr at 37°C with human peripheral blood MNC (Rickles, et al., 1977) and assayed for TF with a one-stage coagulation assay (Rickles, et al., 1979). A clotting time of 30 sec was defined as equal to 100 units TF activity (Korn, et al., 1982).

Endothelial cell tissue factor (TF) assay. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured in media (containing 2% serum) obtained from Clonetics. Cells were grown to confluent monolayers in sterile 96-well tissue culture plate wells (Nunclon, Applied Scientific, San Francisco, CA). E. coli LPS alone or LPS-Hb mixtures were added to the media in each well (final concentrations: 100 ng/ml LPS; 0.1-10 mg/ml Hb), and incubated for 4 hr. Wells were then washed with media (x 3) and the HUVEC were freeze-thawed (x 2) and sonicated in phosphate buffered saline. To each well then was added human citrated plasma and calcium (25 mM), and plates were incubated for 8 min in a temperature-controlled (37°C) plate reader (Kinetic-QLC, Whittaker Bioproducts Inc., Walkersville, MD). Turbidity was measured at 340 nm, and TF activity was calculated from a standard curve established with rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 min by 1:100 diluted thromboplastin was arbitrarily defined as 10 TF units.

RESULTS

Demonstration of LPS-Hb complexes. Ultrafiltration experiments demonstrated that 97% of the LPS in LPS-ααHb mixtures and 94% of the LPS in LPS-HbA_0 mixtures were filterable through the 300 kDa membrane, whereas only 16% of LPS alone was filterable (Fig. 1). Approximately 90% of the total Hb protein in each of these LPS-Hb mixtures, and from filtrates of Hb alone, was detected in filtrates (data not shown). Using 100 kDa cutoff membranes, 64% and 72% of LPS in ααHb or HbA_0 mixtures, respectively, were filterable (data not shown). These results indicated that Hb caused the dissociation of LPS into lower molecular weight particles. Utilizing ethanol precipitation, greater than twice the amount of each Hb was precipitated at 20°C in the presence of LPS than was with Hb alone (Fig. 2). In both the absence and presence of Hb, approximately 90% of LPS was precipitated by ethanol (data not shown). Similar 2-3 fold increases in precipitated Hb in the presence of LPS were demonstrated at 4°C and 37°C (data not shown). These results suggested that Hb and LPS formed stable complexes. Following centrifugation in 5% sucrose, 93% of LPS sedimented into the sucrose cushion (bottom fraction) in the absence of protein, whereas only 9% sedimented in the presence of HbA_0 and 13% in the presence of ααHb (Figure 3). Conversely, only 7% of LPS alone remained above the sucrose layer, whereas in the presence of Hb, 87-91% of LPS remained in the top layer. No detectable Hb entered the sucrose layer in either the absence or presence of LPS. Therefore, Hb decreased the density of LPS, resulting in the co-migration of Hb and LPS. When LPS-ααHb mixtures were centrifuged through a 4-20% sucrose gradient, the two



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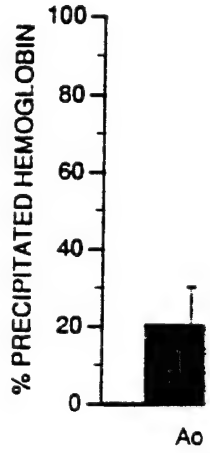

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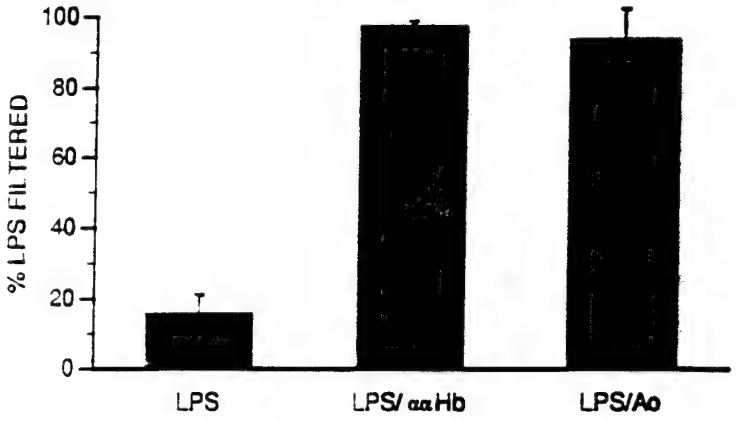

Fig. 1. Ultrafiltration of Hb and LPS. E. coli LPS was incubated with αα Hb or native HbA_O, and the mixtures were then filtered through a 300 kDa cut-off ultrafiltration membrane. The percent of LPS filtered, in the absence and presence of Hb, was determined by the LAL test. Presented are the means and 1 S.D. of 3 experiments. Both of the Hb preparations greatly increased the filterability of LPS.

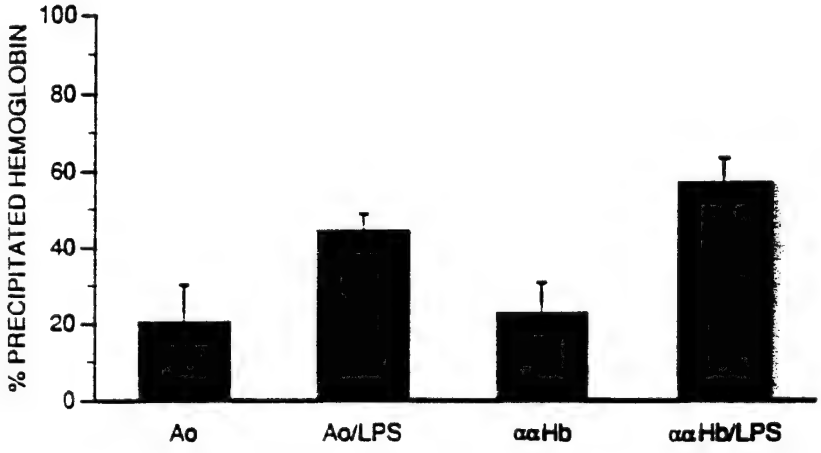

Fig. 2. Precipitation of Hb by ethanol. αα Hb or native HbA_O was incubated with E. coli LPS, and the LPS-Hb complexes or Hb alone were then precipitated from the mixtures by 67% ethanol and sedimented by centrifugation. The quantities of Hb in the sedimented material were determined by protein assays. Presented are the means and 1 S.D. of 8 experiments. Both of the Hb preparations demonstrated increased precipitability in presence of LPS.

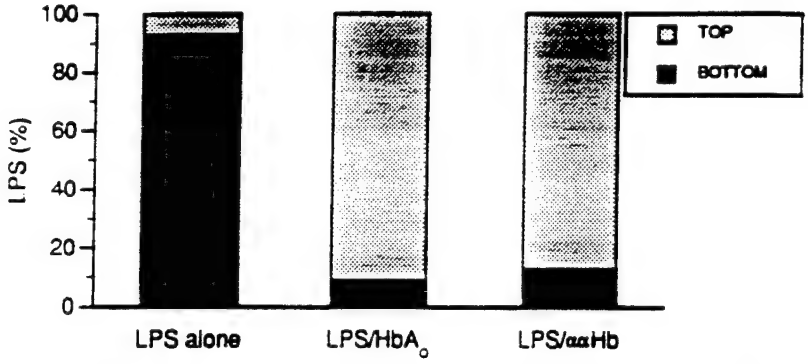

Fig. 3. Centrifugation of Hb and LPS through sucrose. ^{14}C-S. typhimurium LPS was incubated with αα Hb or native HbA_o. These LPS-Hb complexes, or LPS in NaCl, were then centrifuged through 5% sucrose. The distribution of radiolabeled LPS was determined in top (stippled columns) and bottom (solid columns) zones of the centrifuged samples. Presented are the means of 4 experiments. LPS alone distributed predominantly in the bottom zone. LPS in the presence of Hb distributed predominantly into the top zone, indicating a decrease in LPS density. Hb remained in the top zone in the presence or absence of LPS. Both of the Hb preparations co-migrated with LPS.

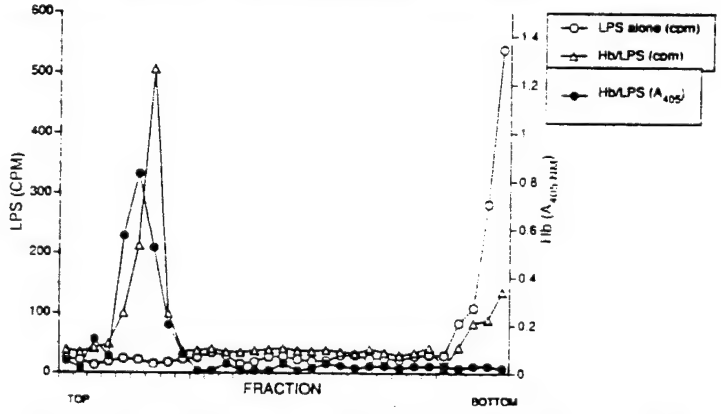

Figure 4. Sucrose density centrifugation of LPS-Hb. ^{14}C-LPS was incubated with αα Hb, and the mixture or LPS alone was centrifuged through a 4-20% continuous sucrose gradient. 0.4 ml fractions were assayed for hemoglobin by absorbance at 405 nm (●), and for LPS by scintillation counting (○, LPS alone; △, LPS in LPS-Hb complexes). The density of LPS was decreased in the presence of Hb, and Hb and LPS co-migrated by density.

components co-migrated part way through the gradient, whereas LPS alone sedimented to the bottom of the tube (Fig. 4). Hb alone sedimented at a rate similar to the Hb-LPS

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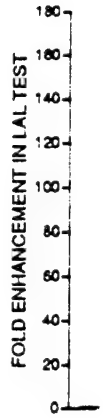

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Biological activity of LPS in Hb-LPS complexes. Hb increased the biological activity of LPS in three independent assays. Firstly, LPS in the presence of ααHb produced enhanced activation of LAL (33-180 fold) compared to LAL activation by LPS alone (Fig. 5). The enhanced LPS biological activity was most prominent with the smooth

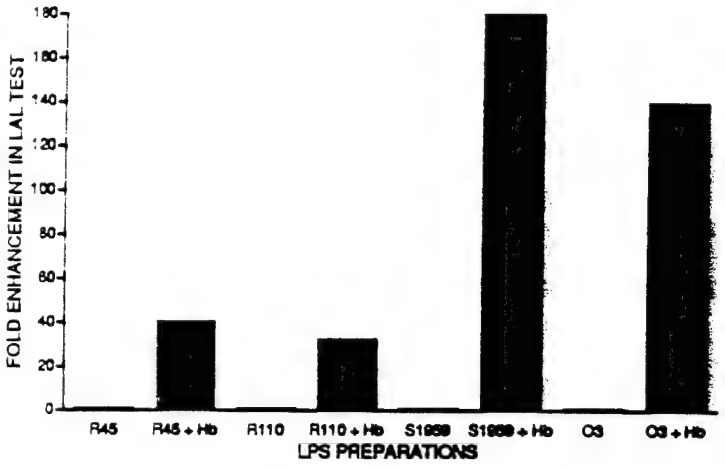

Fig. 5. Enhancement of LPS activation of Limulus amebocyte lysate by Hb. LPSs from P. mirabilis isolates (deep rough R45, rough R110, and smooth S1959 and O3) were incubated with LAL, in the presence and absence of ααHb, and relative LAL reactivities were determined. The reactivity of each LPS alone has been normalized to 1 in order to compare Hb enhancement effects. Presented are the means of 8 experiments. Hb dramatically enhanced the biological activity of each LPS.

Proteus LPSs S1959 and 03, although substantial enhancement was also demonstrated with the rough (R110) and deep rough (R45) mutants. Enhancement of the biological activities of these LPSs also was observed with HbA_0, and the enhancement effect of each Hb was concentration dependent (data not shown). Secondly, LPS-ααHb complexes resulted in greater TF production by human MNC than from MNC following incubation with LPS alone (Fig. 6). The enhancement in TF production was Hb concentration-dependent, ranging from 2-fold at 0.6 mg/ml Hb to 22-fold at 60 mg/ml Hb. Thirdly, ααHb produced an increase in HUVEC TF activity compared to the TF generated by LPS alone, as demonstrated by the increased rate of production of turbidity (absorbance at 340 nm)(Fig. 7). The enhancement effect was Hb concentration-dependent, and LPS-induced

TF activity, as quantified using brain thromboplastin in the standard curve, increased 8-fold in the presence of 10 mg/ml αα-Hb. The enhancement effect was totally inhibited by

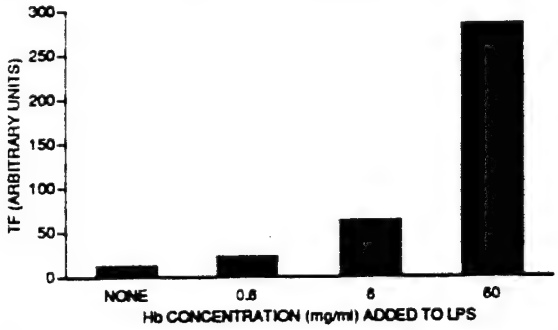

Fig. 6. Effect of Hb on the LPS-induced stimulation of tissue factor procoagulant activity from human peripheral blood mononuclear cells. E. coli LPS (100 ng/ml), in the absence or presence of various concentrations of αα-Hb, was incubated with human mononuclear cells, and tissue factor activity was measured with a plasma clotting assay. Hb enhanced, in a concentration-dependent manner, LPS-stimulated TF production.

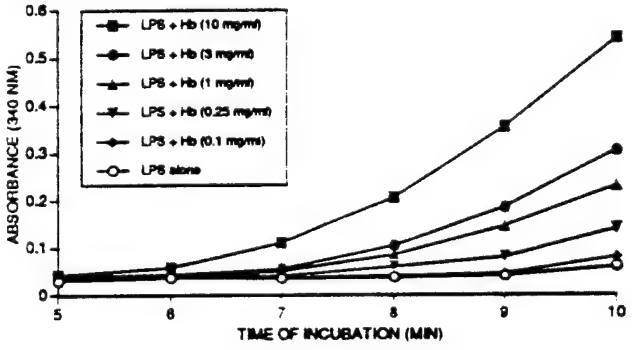

Fig. 7. Effect of LPS on the production of tissue factor procoagulant activity by cultured human endothelial cells. Monolayers of cultured human umbilical vein endothelial cells were incubated with E. coli LPS (100 ng/ml) for 4 hrs in the absence or presence of various concentrations of αα-Hb. The cells were then washed and freezed/thawed twice. Normal human citrated plasma and calcium were added, and clot formation was measured as increased turbidity (A_{340nm}). Tissue factor procoagulant activity was detected as an increased rate of increase in turbidity compared to the clotting rate of recalcified plasma alone. Hb caused a concentration-dependent increase in LPS-stimulated endothelial cell tissue factor activity.

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protein synthesis inhibitors cycloheximide and actinomycin D (data not shown). Similar Hb concentration-dependent enhancement of LPS-induced TF in HUVEC was observed with HbA_0 (data not shown). LPS-induced TF protein, as measured with a sensitive ELISA assay, similarly was enhanced by Hb in a concentrtion-dependent manner.

DISCUSSION

In order to investigate the potential role of LPS contamination in the observed toxicities of infused Hb, we performed experiments to determine whether Hb interacted with LPS. Ultrafiltration demonstrated that the molecular weight of LPS (typically $>10^6$ in aqueous solution) was reduced to < 300 kDa in the presence of Hb, and that LPS and Hb co-filtered. The majority of LPS (64-72%) also was <100 kDa in the presence of Hb. Utilizing centrifugation through sucrose, we showed that the density of LPS in the presence of Hb was distinctly less than that of LPS alone, and that LPS and Hb co-migrated. The decrease in LPS density is further evidence for LPS disaggregation. Measurement of Hb precipitation by ethanol indicated that LPS greatly increased the precipitability of Hb, a result which further provided evidence of complex formation. Therefore, our experiments demonstrated that the physical characteristics of both Hb and LPS were altered in LPS-Hb mixtures. These results are consistent with the formation of stable complexes, and establish the ability of hemoglobin to act as an endotoxin-binding protein. Because these results were observed with both crosslinked Hb ($\alpha\alpha$Hb) and unmodified hemoglobin (A_0), we have demonstrated that LPS-binding is an intrinsic property of hemoglobin.

The formation of LPS-Hb complexes was associated with major changes in the procoagulant activities of LPS. Hb enhanced the ability of LPS to initiate coagulation as demonstrated with three independent assays: 1) direct activation of a proteolytic coagulation cascade, as shown with Limulus amebocyte lysate, 2) stimulation of TF production from human MNC, and 3) stimulation of TF production from HUVEC. Enhancement by Hb of LPS procoagulant activity by one or all of these mechanisms may contribute to the toxicities associated with Hb infusions in resuscitation experiments. This enhanced procoagulant activity of LPS may be the etiology of the observed thrombosis and ischemic damage associated with Hb infusion in animals (Feola, et al., 1988a; Marks, et al., 1989), and may also provide a mechanism for some aspects of the synergistic toxicity between Hb and LPS reported previously (White, et al., 1986b; Litwin, et al., 1963). Interestingly, other proteins that are known to bind LPS with a resultant change in LPS biological activity, e.g., melittin (David, et al., 1992), lysozyme (Ohno, Morrison, 1989), complement (Galanos, et al., 1971) or the polypeptide polymyxin B (Morrison, Jacobs, 1976), cause a decrease in LPS toxicity.

In contrast to the increased biological activity we observed for LPS that had been disaggregated and bound to Hb, the process of LPS disaggregation in plasma resulting primarily from its interaction with high density lipoproteins (Ulevitch, et al., 1979) results in detoxification. It is possible that the process of LPS-Hb complex formation might compete in vivo with the process of the LPS-lipoprotein interaction, and therefore potentially interfere with LPS detoxification in plasma. The combination of decreased detoxification of LPS in plasma and enhancement of LPS biological activity secondary to binding to Hb and disaggregation of LPS micelles would magnify the consequences of endotoxemia. In addition to accounting for some of the toxicity observed with Hb infusions, the Hb-LPS interaction also may provide a mechanism by which LPS-induced

intravascular hemolysis during sepsis potentiates the pathophysiologic consequences of endotoxemia.

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BACTERIAL ENDOTOXINS

Basic Science to Anti-Sepsis Strategies

Proceedings of the Fourth International Conference on Endotoxins (ICEA IV), Held in Amsterdam, The Netherlands, August 1993

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1994

Hemoglobin Enhances the Production of Tissue Factor by Endothelial Cells in Response to Bacterial Endotoxin

By Robert I. Roth

Human endothelial cells respond to bacterial endotoxin (lipopolysaccharide [LPS]) with changes that transform the endothelium into a surface with prominent procoagulant properties. Production of tissue factor (TF) in response to LPS is a major alteration that favors coagulation. Biologic activities of LPS have previously been shown to be enhanced by the presence of hemoglobin. Therefore, the ability of human hemoglobin (Hb) to modulate TF production by cultured human umbilical vein endothelial cells (HUVEC) was investigated. Cell-free Hb (10 mg/mL), either purified native (HbA_o) or chemically cross-linked ($\alpha\alpha$Hb), was incubated with LPS (0.1 μg/mL), and the mixtures then were added to HUVEC in culture. TF activity was quantified with a clotting assay and TF protein was measured with an enzyme-linked immunosorbant assay. Hb preparations greatly enhanced the production of TF activity (11- to 25-fold greater than TF produced by HUVEC alone) compared with minimal TF activity generated by LPS alone (only twofold greater than HUVEC alone). The enhancement of LPS-induced TF activity was Hb concentration-dependent over a range of 1 to 100 mg/mL. Cross-linked $\alpha\alpha$Hb also greatly enhanced the production of TF protein compared with TF protein generated by LPS alone (12-fold greater *v* 3.5-fold greater than HUVEC alone, respectively). The enhancement of LPS-induced TF protein was Hb concentration-dependent over a range of 0.1 to 2 mg/mL. Enhancement of TF activity by Hb required new protein synthesis. These results show that human Hb can augment the ability of LPS to induce endothelial cell TF and suggest that hemolysis associated with disseminated intravascular coagulation during sepsis may further stimulate coagulation. In addition, these results suggest a potential mechanism for generalized thrombosis in animals that has been associated with the infusion of cell-free Hb for resuscitation.

HUMAN ENDOTHELIAL CELLS have a major role in the control of hemostasis. Under normal conditions, the endothelium provides an antithrombotic barrier. Contributing to thromboresistance are the expression of anticoagulant factors such as protein S, thrombomodulin and plasminogen activators,[1,2] presentation of heparin-like molecules,[3] and the inhibition of platelet aggregation by prostacyclin and endothelium-derived relaxing factor.[4] Recently, an inhibitor of the contact activation of coagulation via Hageman factor also has been described.[5] During gram-negative bacterial sepsis, the presence of bacterial lipopolysaccharide (LPS) results in prominent changes involving the endothelium. Endothelial cells exposed to LPS show overall prothrombotic properties, principally by the synthesis and expression of tissue factor (TF),[6,7] the upregulation of plasminogen activator inhibitor,[8] the down regulation of thrombomodulin, and the inhibition of factor C activation.[7] Leukocyte adhesion is enhanced in the presence of LPS,[9,10] and when human umbilical vein endothelial cells (HUVEC) are incubated with thrombin, a coagulation protease that is commonly generated during endotoxemia, adherence of platelets to HUVEC is increased.[11] These endothelial cell changes in response to LPS and the subsequent prothrombotic activities of the endothelium contribute to the multiple organ failure that is one of the prominent pathologic consequences of gram-negative sepsis.

Cell-free hemoglobin (Hb) can be released from erythrocytes during sepsis as a result of coagulation-mediated intravascular hemolysis or bacterial hemolysin activity.[12,13] Plasma Hb levels were reported to be 1 to 2 mg/mL in patients with intravascular hemolysis,[14] and plasma Hb levels up to 2 mg/mL have been reported in rabbits with enzyme-mediated hemolysis[15] or endotoxin-mediated intravascular hemolysis.[16] In these studies, released Hb was in excess of the binding capacity of haptoglobin and was detected as free Hb for several hours. Therefore, LPS and considerable concentrations of Hb may coexist in the blood stream during pathologic conditions, and in previous studies in our laboratory, it has been shown that the two molecules form Hb-LPS complexes.[17] In vitro studies and in vivo observations have suggested that significant pathophysiologic consequences result from the biochemical interaction(s) between Hb and LPS. Hb has been shown in vitro to enhance LPS-initiated activation of coagulation[18] and to stimulate production of mononuclear cell TF.[19] In vivo, synergism of the toxicities of Hb and LPS (eg, synergistic activation of coagulation[20,21] and complement[21] cascades) has been shown, suggesting that enhancement of LPS activity by Hb may contribute to the observed thrombosis, ischemic damage, and multiple organ failure associated with Hb infusion in animals. Therefore, Hb-LPS interactions, resulting from sepsis-mediated hemolysis, may potentiate the deleterious effects of LPS. In addition, the interaction between Hb and LPS in vivo, with resultant synergism of their pathophysiologic effects, constitutes a major potential limitation in the use of Hb-based oxygen carriers for resuscitation.[22] In the present

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study, the effect of Hb on the production of TF by endothelial cells in response to bacterial endotoxin was examined.

MATERIALS AND METHODS

Reagents. Actinomycin D and cycloheximide were obtained from Sigma (St Louis, MO). Sterile tissue-culture plasticware was obtained from Becton Dickinson (Mountain View, CA).

Hb. Purified human hemoglobin A_0 (HbA_0), prepared by ion exchange high pressure liquid chromatography of purified human Hb, as described previously,[23] was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR; San Francisco, CA). Human cross-linked cell-free hemoglobin ($\alpha\alpha$Hb), covalently cross-linked between the two α-chains with bis(3,5-dibromosalicyl) fumarate as described previously,[24] also was provided by collaborators at BRD/LAIR. These Hb preparations contained less than 0.4 EU/mL endotoxin (referenced to *Escherichia coli* LPS B, O55:B5; Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate test,[25] and did not contain demonstrable erythrocyte stroma, as shown by phosphorus analysis and reverse-phase high pressure liquid chromatography. Hb concentrations were determined spectrophotometrically.

Endotoxin. *E coli* O26:B6 (W) LPS was purchased from Difco Laboratories.

HUVEC. HUVEC and endothelial cell culture media containing 2% fetal bovine serum, 10 ng/mL epidermal growth factor (EGF) and 1 ng/mL hydrocortisone were purchased from Clonetics (San Diego, CA). HUVEC were plated in 96-well microtiter plates (Nunclon; Applied Scientific, South San Francisco, CA) at a seeding density of 3,500 cells/cm^2 (5,000 cells/well) and were grown to confluency at 37°C and 5% CO_2. HUVEC were used at less than 6 passages.

TF procoagulant assay. TF activity was quantified with a plasma recalcification assay. Confluent HUVEC monolayers in 96-well plates were incubated for 4 hours at 37°C with 10 μL *E coli* LPS alone, HbA_0 or $\alpha\alpha$Hb alone, or Hb-LPS mixtures in 90 μL media/well. Standard incubations were conducted using 10 mg/mL Hb. Experiments were performed with 6 to 8 replicate wells. Wells were then washed with media (×3) and the HUVEC were freeze-thawed twice and sonicated in 50 μL phosphate-buffered saline for 2 minutes at room temperature. To each well then was added 50 μL normal human citrated plasma and 50 μL calcium (25 mmol/L), and after 8 minutes, turbidity was measured at 340 nm in a 37°C temperature-controlled plate reader (Kinetic-QLC; Whittaker Bioproducts Inc, Walkersville, MD). TF activity was calculated from the turbidity generated in plasma (the mean from 6 to 8 replicate wells) by a standard curve established with dilutions of rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 minutes by 1:100 diluted thromboplastin was defined as 1 TF arbitrary unit.

TF protein assay. Confluent HUVEC monolayers in 96-well plates were incubated for 4 hours at 37°C with 10 μL *E coli* LPS alone, HbA_0 or $\alpha\alpha$Hb alone, or Hb-LPS mixtures in 90 μL media/well. Standard incubations were conducted using 2 mg/mL Hb. Experiments were performed with 6 to 8 replicate wells. Wells were then washed with media (×3), and the HUVEC were freeze-thawed twice and sonicated in 50 μL phosphate buffered saline; 10 μL Triton X-100 was added (final concentration, 1%), and incubations were continued overnight at 4°C. The solutions were then removed from the plates and centrifuged for 15 minutes (Microfuge B; Beckman Instruments, Inc, Palo Alto, CA). TF was quantified by enzyme-linked immunosorbant assay (ELISA) using a murine antihuman TF monoclonal antibody (Imubind; generously provided by American Diagnostica, Inc, Greenwich, CT) according to the manufacturer's directions.

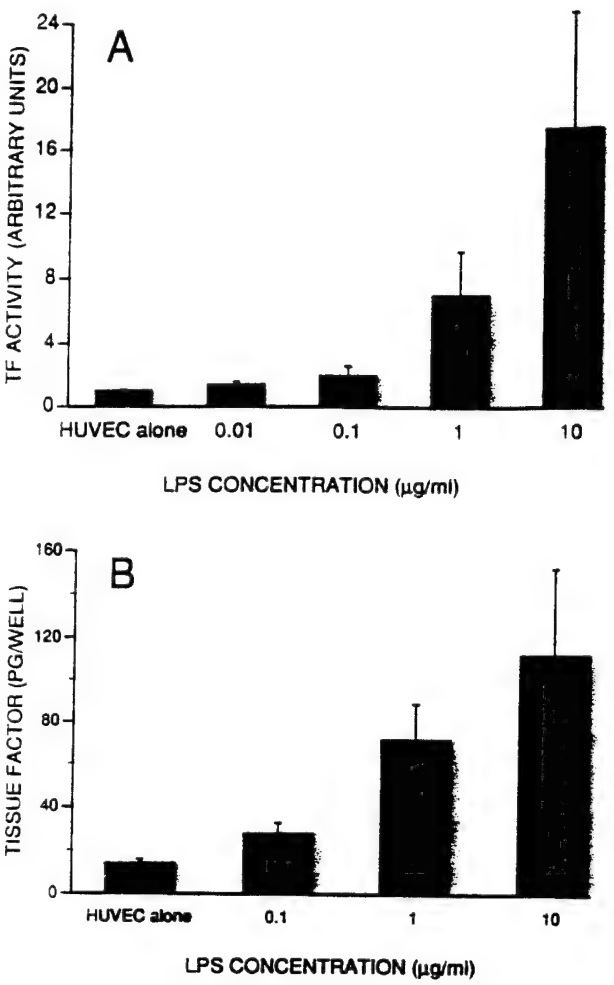

Fig 1. TF produced by HUVEC in response to LPS is shown. Various concentrations of *E coli* LPS were incubated with HUVEC for 4 hours, and TF activities (A) and TF protein concentrations (B) were determined. (A) TF activities, determined with a plasma recalcification assay, were normalized to 1 for TF production by HUVEC alone. The means and 1 SD of 8 tissue culture-plate wells are presented. (B) TF production, expressed as picograms of TF per tissue culture-plate well, was determined with an ELISA assay. The means and 1 SD of 6 wells are presented.

RESULTS

HUVEC contained undetectable or very low TF activity (<0.1 TF unit), as determined by the plasma recalcification assay. After incubation with *E coli* LPS, a concentration-dependent and time-dependent stimulation of HUVEC TF production was observed. In a representative experiment, stimulation of TF activity ranged from 1.4-fold over baseline, after 4-hour incubation with 0.01 μg/mL LPS, to 17.5-fold over baseline, after incubation with 10 μg/mL LPS (Fig 1A). At 100 μg/mL or 1 mg/mL LPS, TF production in some experiments was less than the TF produced by 10 μg/mL LPS and, in other experiments, was equivalent. The increase in TF was detectable after 2 hours of incubation with LPS and became maximal between 4 and 6 hours (data not

Table 1. Relative Procoagulant Activities of Chemically Different Glycolipids

Lipid A*	Deep Rough LPS Mutant†	Rough LPS Mutant‡	Rough LPS Mutant§	Smooth LPS	Smooth LPS¶
1	3	8	18	4	6

Various types of purified LPS were incubated with HUVEC for 4 hours, and TF activities were determined. The LPS that elicited the least TF response by the HUVEC (*S minnesota* lipid A) was assigned a relative procoagulant activity of 1, and the biologic activities of the other LPSs are presented in comparison to lipid A.

* *S minnesota* lipid A.

† *S minnesota* 595.

‡ *Protens mirabilis* R110.

§ *P mirabilis* R45.

P mirabilis S1959.

¶ *E coli* O55:B5.

shown). Similar LPS concentration-dependent TF responses were detected in 8 independent experiments: the maximal TF activities from LPS-treated HUVEC (achieved with 10 μg/mL LPS) were from 7 to 28 times greater (mean, 16 times greater) than the TF activity of untreated HUVEC. TF responses varied considerably when different glycolipids were examined (Table 1). A low TF response was observed with *S minnesota* lipid A (which consists of a diglucosamine backbone and seven fatty acyl chains) compared with other LPSs which contained additional saccharide moieties, suggesting that O-chain and core carbohydrates are important for the HUVEC response. We also quantified HUVEC TF protein by a sensitive ELISA assay. In an experiment representative of 6 independent studies, TF protein increased in an LPS concentration-dependent manner from 17 pg/well in untreated HUVEC to 116 pg/well in HUVEC after 4 hr incubation with 10 μg/ml LPS (Fig 1B). Total protein per well was unchanged at 41 ± 4 μg/well. In each of the 6 studies, LPS-treated HUVEC showed both increased TF functional activity and increased antigenic concentrations of TF protein.

To further demonstrate that the TF activity induced by LPS resulted from newly formed protein rather than by a process of enhanced catalytic activity by preexisting TF protein, we examined the effect of protein synthesis inhibitors. LPS-stimulated TF production was completely inhibited by actinomycin D or cycloheximide (Fig 2), providing additional evidence that the cellular procoagulant response to LPS required new protein synthesis.

To test the effect of Hb on the production of HUVEC TF in response to LPS, two Hb preparations were investigated. HbA_o was used because this preparation was native Hb and would be potentially available to interact with the endothelium during in vivo hemolysis of erythrocytes. $\alpha\alpha$Hb was used because this chemically stabilized preparation is not susceptible to dissociation of the α-chains of the Hb tetramer[6] and is a form of Hb that is presently being developed as a cell-free blood substitute. Hb (10 mg/mL) was first incubated with LPS for 30 minutes at 37°C, and the mixtures then were added to HUVEC. A low concentration of LPS

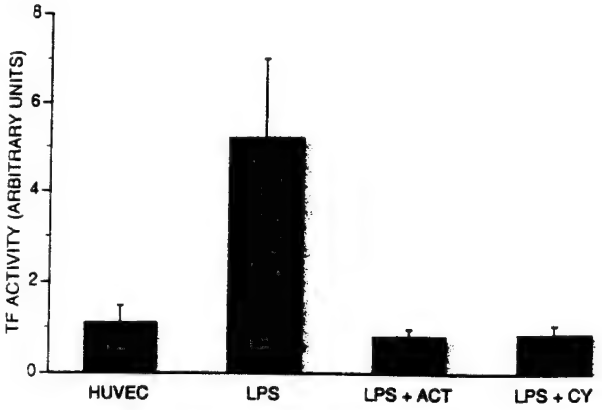

Fig 2. Effect of protein synthesis inhibitors on production of TF by HUVEC in response to LPS is shown. HUVEC were incubated with *E coli* LPS (1 μg/mL) for 4 hours in the absence or presence of actinomycin D (ACT) or cycloheximide (CY; each 10 μg/mL), and TF activities then were determined with a plasma recalcification assay. The means and 1 SD of 8 tissue culture-plate wells are presented.

was used (0.1 μg/mL) so that only modest TF production by HUVEC was generated in response to LPS alone. At this concentration, HUVEC incubated with LPS generated only 2.5-fold greater TF activity than unstimulated HUVEC (Fig 3). HbA_o or $\alpha\alpha$Hb alone (10 mg/mL each) did not demonstrably increase the very low levels of TF activity produced by HUVEC in the absence of LPS. In contrast, stimulation of TF production from HUVEC was 11-fold increased in the presence of the LPS-HbA_o mixture and was 25-fold increased in the presence of the LPS-$\alpha\alpha$Hb mixture. Preincubation of Hb and LPS longer than 30 minutes before their addition to HUVEC did not alter this response.

The enhanced TF production in the presence of various concentrations of Hb was concentration-dependent, as is

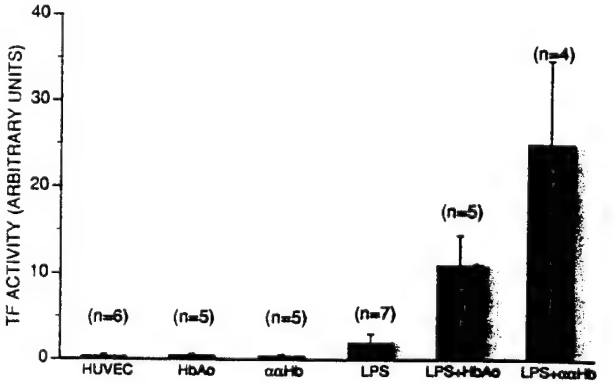

Fig 3. TF activity produced by cultured HUVEC in the presence of LPS and LPS-Hb mixtures is shown. HUVEC were incubated with *E coli* LPS (0.1 μg/mL) for 4 hours in the absence or presence of HbA_o or $\alpha\alpha$Hb (10 mg/mL each). TF activities were determined with a plasma recalcification assay. The means and 1 SD of 4 to 7 independent experiments (n) are presented. For each independent experiment, TF activity was determined from the average of 6 tissue culture wells.

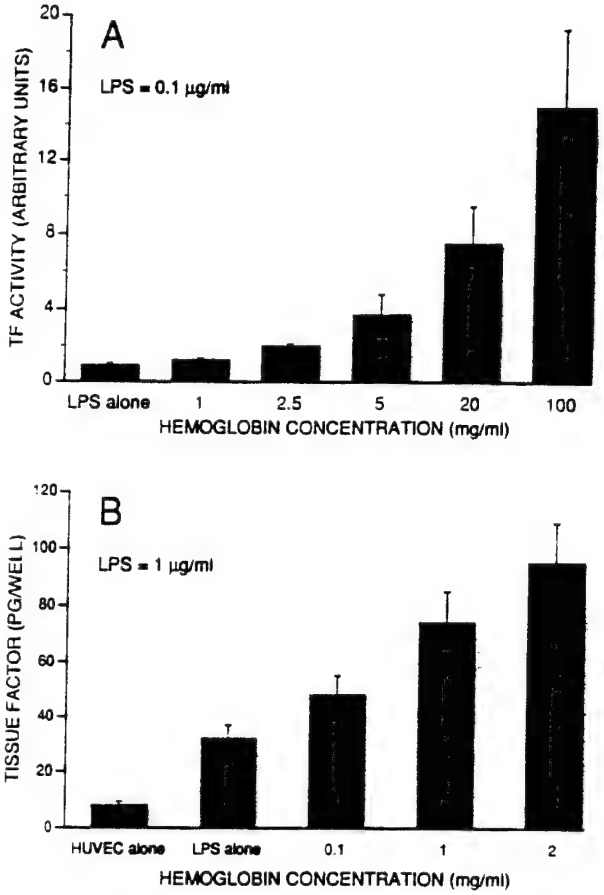

Fig 4. Enhanced production of TF by HUVEC in the presence of LPS-Hb is shown. (A) *E coli* LPS (0.1 μg/mL) was incubated with various concentrations of HbA_o and the mixtures, or LPS alone, then were added to HUVEC in culture. After incubation for 4 hours, TF activity was quantified by a plasma recalcification assay. TF activities were normalized to 1 for TF production by HUVEC in response to LPS alone. (B) HUVEC were incubated with *E coli* LPS (1 μg/mL) alone or with LPS in the presence of various concentrations of ααHb. TF production, expressed as picograms of TF per tissue culture-plate well, was determined with an ELISA assay. The means and 1 SD of 6 wells are presented.

shown for HbA_o (Fig 4A). A similar response to increasing concentrations of HbA_o was observed when experiments were performed with ααHb (data not shown). Supernatants of the HUVEC cultures did not contain detectable TF activity, even after incubation with LPS or LPS-Hb mixtures (data not shown). ELISAs showed Hb concentration-dependent synthesis of new TF protein in response to LPS-Hb mixtures (Fig 4B). In this representative experiment, TF protein production was stimulated 3.9-fold over baseline by 1 μg/mL LPS (in the absence of Hb) and 11.8-fold over baseline in the presence of LPS and 2 mg/mL ααHb. This represents a threefold enhancement in TF protein production because of ααHb. In 4 independent experiments, maximum TF concentrations (pg/well) produced in response to Hb-LPS mixtures were 2.1- to 6.5-fold greater (mean, 3.7-fold) than the concentrations of TF protein produced in response to LPS alone.

To determine whether the enhancement of LPS-elicited TF production in the presence of Hb was the result of new TF protein production, HUVEC were incubated with LPS and Hb in the presence of actinomycin D or cycloheximide. Each of the protein synthesis inhibitors totally prevented the generation of TF activity, indicating that the mechanism for the Hb enhancement process involved new protein synthesis (Fig 5).

DISCUSSION

Despite the extensive existing knowledge of the structure of human Hb and its function within the erythrocyte, relatively little is known about pathophysiologic interactions involving extraerythrocytic Hb and other blood elements and host tissues. Clinical evidence of renal, vascular, and reticuloendothelial system damage during hemolytic episodes has suggested that extraerythrocytic Hb (and, in particular, Hb breakdown products such as hematin) can be toxic.[27] However, the direct effects of Hb have been difficult to determine because the contribution to the observed toxicities from erythrocyte membrane components is also felt to be of major importance.[21,28] Similarly, in determining the mechanism of organ toxicity associated with LPS-mediated intravascular coagulation with resultant hemolysis, it is difficult to distinguish the contribution of Hb to the observed deleterious effects from the contribution by LPS.[20,21,28] The elucidation of LPS-mediated toxicity itself is difficult because of the high potency and the protean biologic activities of LPS. The potential for LPS and cell-free Hb to interact in the blood stream adds additional complexity to an understanding of the consequences of extraerythrocytic Hb.

The current efforts to develop cell-free Hb as a transfusion product have allowed detailed investigation of the interactions between Hb and LPS. Previously, we had shown that there is a biologically significant interaction between Hb and

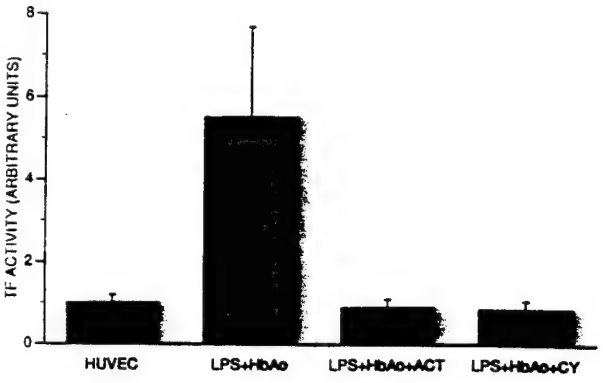

Fig 5. Effect of protein synthesis inhibitors on production of TF by HUVEC in response to LPS-Hb mixtures is shown. *E coli* LPS (1 μg/mL) was incubated with HbA_o (10 mg/mL), and the mixtures, or LPS alone, then were incubated with HUVEC for 4 hours in the absence or presence of actinomycin D (ACT) or cycloheximide (CY; each 10 μg/mL). After incubation, TF activities were determined with a plasma recalcification assay. The means and 1 SD of 8 tissue culture-plate wells are presented.

LPS, with resultant activation of host effector mechanisms. In those studies, mixtures of Hb and LPS were shown to synergistically activate human mononuclear cells and enzymatic coagulation mechanisms.[17] LPS biologic activity in these model systems was clearly shown to be enhanced by Hb. Therefore, modification of LPS by Hb is a process with substantial clinical relevance. In addition, we have recently shown that Hb and LPS form bimolecular complexes and have shown that large LPS aggregates are dissociated on binding to Hb.[17]

Because of the prothrombotic actions of LPS on the vascular endothelium, it was important to investigate the potential ability of Hb to modify this critical interaction during endotoxemia. The present studies showed that HbA_o significantly increased TF activity in HUVEC in response to LPS. This effect was observed at concentrations of Hb (1 to 2 mg/mL) that can be encountered in plasma during clinical and experimental endotoxemia.[14-16] This Hb preparation did not contain detectable erythrocyte stroma that could potentially elicit a HUVEC response. The enhanced TF activity resulted from new TF protein synthesis. The mechanism of this effect is uncertain because the mechanism of LPS signal transduction in HUVEC is not known. However, the demonstration in our laboratory that Hb binds LPS and causes LPS dissociation[17] suggests that disaggregated and/or protein-bound LPS has an increased ability to interact with HUVEC LPS receptors and trigger the procoagulant response. Thus, the Hb enhancement effect may represent the result of presentation to the endothelial cell of a "modified" (eg, disaggregated) LPS. Additionally, a number of other potential mechanisms may be involved. Preliminary studies in our laboratory have shown that LPS induces the denaturation of Hb to methemoglobin and hemichrome, species of Hb that may show altered biologic activity and produce oxygen-free radicals during their formation.

Based on these results using HbA_o, it is reasonable to propose that hemolysis caused by LPS-mediated disseminated intravascular coagulation may constitute a positive feedback loop to amplify coagulation. Additionally, enhancement of other LPS biologic activities by HbA_o, such as mononuclear cell cytokine production, may contribute to the often fatal consequences of low level endotoxemia (during which LPS concentrations are frequently measured in the 10 to 100 pg/mL range[29,30]). The present study also showed enhancement of the TF response to LPS by $\alpha\alpha$Hb, a cross-linked preparation of human Hb that is a leading candidate for use as a blood substitute. These findings raise two concerns for the human use of cell-free Hb. Firstly, contamination of Hb preparations by environmental LPS is difficult to avoid.[19] Because large volumes of Hb would be required for resuscitation, the procoagulant consequences of LPS contamination in the Hb preparations could limit the use of $\alpha\alpha$Hb. In patients receiving 1/10th blood volume of $\alpha\alpha$Hb for resuscitation, blood Hb levels $\geq$10 mg/mL would be achieved. At these concentrations of Hb, substantial enhancement by Hb of the procoagulant activity of any LPS in the circulation would be expected based on our results. Secondly, $\alpha\alpha$Hb would likely be infused into some patients with coexisting endotoxemia. Endotoxemia could arise from gram-negative bacteremia, from reticuloendothelial system dysfunction, and/or from increased gastrointestinal tract translocation of LPS into the circulation secondary to hypotension or trauma. Consequently, $\alpha\alpha$Hb infusion could potentially enhance the systemic pathologic effects of underlying endotoxemia. Because of the demonstration of the in vitro consequences of the interaction between LPS and Hb, which provide a basis for potential thrombotic effects, it is important that future studies investigate the ability of Hb to enhance LPS-induced coagulation in vivo.

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Hemoglobin, a Newly Recognized Lipopolysaccharide (LPS)-binding Protein That Enhances LPS Biological Activity*

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Cell-free hemoglobin (Hb) is a purified preparation of human hemoglobin that is being developed as a resuscitation fluid. *In vivo* administration of hemoglobin has resulted in significant toxicity, due in part to contamination with bacterial endotoxin (lipopolysaccharide (LPS)). To better understand this toxicity, we have studied the interaction between Hb and LPS. Mixtures of each of three different Hb preparations (cross-linked αα-Hb, cross-linked carbon monoxy-αα-HbCO, and noncross-linked (native) HbA_0) and LPS (*Escherichia coli* O26:B6 or *Proteus mirabilis* S1959) were examined by several independent methods for evidence of Hb·LPS complex formation. Binding assays in microtiter plates demonstrated saturable binding of LPS to immobilized Hb, with a k_D of 3.1×10^{-8} M. Binding of LPS to Hb also was demonstrated with a radiolabeled LPS photoaffinity probe. Ultrafiltration of Hb/LPS mixtures by 300- and 100-kDa cut-off membranes showed that the majority of LPS in these mixtures (87–97 and 64–72%, respectively) was detected in the filtrates, in contrast to the lack of filterability of LPS in the absence of Hb. Density centrifugation demonstrated that LPS co-migrated with each of the three Hbs, whereas unbound LPS had a distinctly greater sedimentation velocity than Hb or Hb·LPS complexes. Nondenaturing polyacrylamide gel electrophoresis demonstrated that in the presence of Hb, LPS migrated into the gel and co-electrophoresed with Hb, whereas LPS alone did not appreciably enter the gel. Finally, precipitation by ethanol of each of the three Hb preparations was increased in the presence of LPS compared with precipitation in the absence of LPS. Interaction of LPS with each of the three Hb preparations was also associated with altered biological activity of LPS, as shown by enhancement of LPS activation of *Limulus* amebocyte lysate. Therefore, our data provide several lines of independent evidence for Hb·LPS complex formation and indicated that LPS exhibited altered physical characteristics and enhanced biological activity in the presence of Hb.

Cell-free hemoglobin (Hb)[1] is a preparation of human hemoglobin that is being developed for use as an oxygen-transporting resuscitation fluid (1, 2). Hb has excellent oxygen delivery properties and a long shelf life and, therefore, is a potentially ideal red blood cell substitute. However, Hb has not yet been used clinically because of significant problems of toxicity. Hypertension and bradycardia have been commonly observed (3, 4), and a decrease in glomerular filtration rate and renal plasma flow have been described (5). Mild prolongations of the partial thromboplastin time also have been reported (4). In some animal studies, preparations of Hb have been shown to produce fever, disseminated intravascular coagulation with resultant thrombosis, and ischemic parenchymal damage (6, 7).

Whether the reported toxicity is due to hemoglobin, *per se*, or to contaminants such as stromal phospholipids or bacterial endotoxin (lipopolysaccharide, LPS) is still uncertain, and inconsistent results have been described. Widespread parenchymal organ damage and activation of the complement and coagulation cascades have been demonstrated in hemoglobin preparations that contained detectable stromal phospholipids (6–8). Similarly, increased lethality in rabbits that received Hb contaminated with LPS, compared with Hb in the absence of detectable LPS, has indicated a role for endotoxin in causing *in vivo* toxicity of Hb (9, 10). In contrast, hepatotoxicity has been reported in the absence of detectable LPS or stromal lipid (7), thus suggesting intrinsic hemoglobin toxicity.

Because it remains unknown whether Hb binds LPS, and since binding could alter the biological activity of LPS, the present study was designed to evaluate the interaction between these molecules. Our data indicate that complex formation occurs between Hb and LPS, and that the procoagulant activity of Hb·LPS is increased compared with LPS alone.

MATERIALS AND METHODS

Reagents—Falcon centrifuge tubes (sterile, 15 ml) were obtained from Becton Dickinson (Mountainview, CA). Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL). RNase and DNase were purchased from Sigma.

Glassware—All glassware was rendered endotoxin free by heating at 190 °C in a dry oven for 4 h.

Hemoglobin—Human cell-free hemoglobin (Hb), prepared and purified as described previously (11, 12), was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research, San Francisco, CA. Hb cross-linked between α chains with bis(3,5-dibromosalicyl)fumarate (αα-Hb) was 9.6 g/dl (95.4% cross-linked, 96.3% oxyhemoglobin, 3.2% methemoglobin), pH 7.4, in Ringers acetate and contained less than 0.4 endotoxin units/ml (referenced to *Escherichia coli* lipopolysaccharide B, O55:B5, Difco), as determined by the *Limulus* amebocyte lysate (LAL) test (13). The αα-Hb stock solution was stored at −70 °C. Carbon monoxyhemoglobin (αα-HbCO) was produced by incubation of the αα-Hb solution with CO and also was at 9.6 g/dl (95.4% cross-linked, 95% HbCO, and 5% oxyhemoglobin). Purified noncross-linked hemoglobin A_0 (HbA_0), 8.4 g/dl, was prepared as described previously (14).

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[1] The abbreviations used are: Hb, cell-free human hemoglobin; αα-Hb, bis(3,5-dibromosalicyl)fumarate αα-cross-linked cell-free hemoglobin; αα-HbCO, αα-cross-linked cell-free carbonmonoxyhemoglobin; HbA_0, noncross-linked cell-free hemoglobin A_0; LPS, bacterial lipopolysaccharide; LAL, *Limulus* amebocyte lysate; HSA, human serum albumin; PBS, phosphate-buffered saline.

Albumin—Human serum albumin (HSA) 25%, for injection) was purchased from Nybcen (New York, NY).

Endotoxins—*E. coli* O26:B6 (W) and O55:B5 (B) LPS were purchased from Difco. Lipopolysaccharide from *Proteus mirabilis* S1959, purified by sequential treatment with RNase and DNase followed by ultracentrifugation (15, 16), has been described previously (16–18) and was provided by collaborators at the Institute of Microbiology and Immunology, University of Lodz, Poland. ^{14}C-Lipopolysaccharide (*Salmonella typhimurium* PR122(Rc), 1 μCi/mg) was purchased from List Biologicals, Inc. (Campbell, CA). ^{125}I-Lipopolysaccharide (*E. coli* O26:B6, 0.1 μCi/μg) was prepared as described previously (19). *Salmonella minnesota* 595 LPS, Re type, extracted by the phenol/chloroform/petroleum ether method (20), was utilized to prepare the photoaffinity probe ^{125}I-LPS-ASD (*S. minnesota* Re595 LPS-(*p*-azidosalicylamido)-1,3′-dithioproprionamide) as described previously (21).

Limulus Amebocyte Lysate—Amebocyte lysates were prepared from *Limulus polyphemus* (the North American horseshoe crab) by disruption of washed amebocytes in distilled water, as described previously (13, 22).

Chromogenic Substrate—Chromogenic substrate S-2423 (AB Kabi Vitrum, Molndal, Sweden) was the gift of Dr. Petter Friberger and was reconstituted with pyrogen-free water.

Chromogenic LAL Test—Activation of LAL by endotoxin was used 1) to compare the biological activity of LPS in the presence and absence of Hb and 2) to determine LPS concentrations in samples after filtration procedures. Dilutions of endotoxins or endotoxin-containing protein solutions were prepared, using pyrogen-free 0.9% NaCl, in sterile, 96-well, flat bottom Falcon microtest II tissue culture plates (Becton Dickinson, Mountain View, CA). 50 μl of sample and 30 μl of LAL (freshly diluted 1:20 in 0.9% NaCl prior to use) were incubated in tissue culture plates for 30 min at 37 °C in a temperature-controlled plate reader (Kinetic-QCL, Whittaker Bioproducts Inc., Walkersville, MD). 40 μl of chromogenic substrate S-2423 (0.25 mM, in 25 mM Tris, pH 8.6) was then added to each well. Mixtures were incubated at 37 °C for 5 min, and absorbances at 405 nm were determined. Background absorbance at 405 nm (which included a component of absorbance due to Hb) was subtracted from each reading. Samples were assayed in duplicate or triplicate.

Gelation LAL Test—Samples were assayed for Hb or HSA enhancement of the biological activity of LPS with the LAL test using gelation as the end point (13, 22). LPS concentrations in samples were calculated based on a LPS standard curve established with *E. coli* O55: B5.

Binding of LPS to Hb-coated Microtiter Plate Wells—Incubations of LPS and Hb in microtiter plates were utilized to demonstrate binding of LPS to immobilized Hb and determine affinity. ααHb (1 μg/well in phosphate-buffered saline, pH 7.4 (PBS)) was added to each well of a 96-well polyvinyl soft round bottomed microtiter plate and incubated at 37 °C overnight. Wells were then washed 3 times with PBS, and excess binding sites were blocked with 100 μl of bovine serum albumin/well (1 mg/ml). After 2 h, unbound bovine serum albumin was removed with three PBS washes, and 100 μl of various concentrations of ^{125}I-LPS (*E. coli* O26:B6 LPS, 1.7×10^4 cpm/μg) in PBS was added. In control experiments to determine nonspecific binding, ^{125}I-LPS was added to bovine serum albumin-blocked wells in the absence of Hb. Following a 4-h incubation, unbound LPS was removed, and the wells were then washed three times with PBS. The wells were cut from the microtiter plates, and bound ^{125}I-LPS determined in a gamma counter (LKB Automatic Gamma Counter, LKB Instruments, Inc., Gaithersburg, MD). Assays were performed in triplicate wells.

Binding of an LPS Photoaffinity Probe to Hb—^{125}I-LPS-ASD photoaffinity probe (0.1 μCi) (prepared as described above) containing 2 μg of LPS in PBS was incubated in the dark with ααHb (10 μg in PBS) for 30 min at 37 °C. Control incubations contained excess nonradiolabeled *S. minnesota* 595 LPS (200 μg) as a blocking agent to demonstrate inhibition of specific binding. Cross-linking was accomplished by photolysis with shortwave UV irradiation (254 nm) (UVGC-25 lamp; UVP Inc., San Gabriel, CA) at a distance of 1 cm for 15 min. Samples were reduced with 2-mercaptoethanol, electrophoresed in acrylamide in the presence of SDS, and subjected to autoradiography, as described previously (23). Hemoglobin-associated ^{125}I was determined by excising Coomassie Blue-stained protein bands and counting the associated counts/min in a gamma counter.

Ultrafiltration—Ultrafiltrations were performed using XM 100 (100-kDa cut-off) ultrafilters (Amicon Division, W.R. Grace, Danvers, MA) and ultrafree-PFL polysulfone 300 (300-kDa cut-off) ultrafilters (Millipore Corp., Bedford, MA). Filters with holders were washed with pyrogen-free 0.9% NaCl until filtrates had less than 1 ng/ml LPS as determined by the LAL test (see above). 0.9 ml ααHb, ααHbCO, or HbA_0 (diluted to 96, 96, and 84 μg/ml, respectively, with pyrogen-free 0.9% NaCl) was incubated with 0.1 ml *E. coli* O26:B6 (W) or *P. mirabilis* S1959 LPS (each 50 μg/ml in 0.9% NaCl; 5 μg/ml, final concentration) for 30 min at 37 °C. Mixtures then were filtered manually with a 3-ml syringe (according to the directions of the filter manufacturers) at room temperature, using the 300- or 100-kDa cut-off filters. LPS concentrations in filtered solutions of Hb, Hb and LPS mixtures, or LPS alone were determined by the chromogenic LAL test (described above), using starting mixtures of Hb/LPS or LPS alone for the standard curve. Utilization of the starting mixtures for the standard curves corrected for any potential change in LPS biological activity that could occur in the presence of Hb. Hb protein concentrations were determined by the BCA protein assay (Pierce Chemical Co.). The mean values of three filtration experiments are presented.

Sucrose Centrifugation of LPS and Hb—Sucrose (4 or 20% in pyrogen-free water) was rendered endotoxin-free by filtration through an immersible CX-10 (10 kDa cut-off) ultrafiltration membrane (Millipore Corp., Bedford, MA), and 12-ml continuous sucrose gradients (4–20%) were prepared. ^{14}C-Labeled *S. typhimurium* LPS (0.005 μCi) was added to ααHb, and the mixtures were incubated for 30 min at 20 °C. 0.1 ml of the mixture (which contained 0.002 μCi) was layered above the sucrose and centrifuged at $52,000 \times g$ for 4 h in a Sorvall RC70 centrifuge and T641 swinging bucket rotor (DuPont). Following centrifugation, the tubes were then punctured and 0.4-ml fractions were collected. Hb was detected by absorbance at 405 nm. LPS was quantified by scintillation counting after samples were diluted 10-fold in fluor (Formula A-989, DuPont NEN), in a Tracor Analytic Liquid Scintillation System (Tracor Analytic, Elk Grove Village, IL). For samples containing hemoglobin, quenching of ^{14}C-LPS was reversed as follows: 0.1 ml aliquots of fractions were diluted 10-fold in water (to 1 ml, final volume), and 1 ml of Solvable (DuPont NEN) was added. These mixtures were incubated at 60 °C for 1 h, and then 0.3 ml 25% H_2O_2 was added. After 30 min of additional incubation at room temperature, samples were pale yellow in color and could be analyzed for radioactivity. Recovery of spiked radioisotope in preliminary experiments to determine the effectiveness of the decolorizing procedure demonstrated >98% detection of previously added radioactivity.

Unbound LPS also was separated from Hb-LPS complexes (and free Hb) by centrifugation through a fixed concentration of sucrose. ααHb, ααHbCO, HbA_0, and HSA (each diluted to 10 mg/ml) were each added to ^{14}C-labeled *S. typhimurium* LPS (0.005 μCi), and the mixtures were incubated for 30 min at 20 °C. 0.3-ml aliquots of LPS/protein mixtures, LPS alone, or protein alone then were layered over 2 ml of 5% pyrogen-free sucrose and centrifuged at $2,900 \times g$ for 30 min at 20 °C in a Sorvall RC-5 centrifuge (DuPont). Unbound LPS predominantly sedimented to the bottom of the tube under these conditions, whereas proteins (both in the presence and absence of LPS) remained above the sucrose layer. Following centrifugation, the solutions were separated into top (0.7–0.9 ml, including the 0.3-ml sample volume plus approximately 0.5 ml at the sample/sucrose interface), middle (0.3–0.6 ml), and bottom (0.5–0.8 ml) fractions. Hb was detected by absorbance at 405 nm, and LPS was detected by scintillation counting, as described above.

Nondenaturing Polyacrylamide Gel Electrophoresis of Hb/LPS Mixtures—Samples of ^{14}C-LPS (9,000–15,000 cpm total), ααHb (50 μg), or ααHb/LPS mixtures were electrophoresed in the absence of SDS in 12% polyacrylamide gels (24) for 1 h at 200 V. Following electrophoresis, the unstained gel was dried and cut into 3-mm pieces, and then each gel piece was analyzed for Hb by absorbance at 405 nm and for LPS by scintillation counting, as described above.

Ethanol Precipitation of Hb and Hb/LPS Mixtures—Insolubility of LPS in ethanol was utilized to obtain Hb complexed to LPS. 25 μl of ααHb (2.4 μg), ααHbCO (2.4 μg), or HbA_0 (2.1 μg) and 25 μl of *E. coli* O26:B6 (W) or *P. mirabilis* S1959 LPS (25 μg each) were incubated in microtiter plate wells for 30 min at 37, 20, or 4 °C. 100 μl of ethanol (100%) then was added to each well (final concentration, 67%), and the incubations were continued for an additional 30 min at their respective temperatures of incubation (*i.e.* 37, 20, or 4 °C). The mixtures then were centrifuged at $800 \times g$ for 30 min in a Sorvall GLC-2 centrifuge (DuPont), supernatants were removed, and the precipitates were resuspended in 50 μl of 0.9% NaCl. The concentrations of Hb in the resuspended sediments were determined by protein assay, and LPS concentrations in the resuspended precipitates were determined by the phenol-concentrated H_2SO_4 method (25), with galactose as the standard.

RESULTS

Binding of LPS to Hb-coated Microtiter Plate Wells—LPS bound to Hb-coated wells in a saturable manner (Fig. 1). Bind-

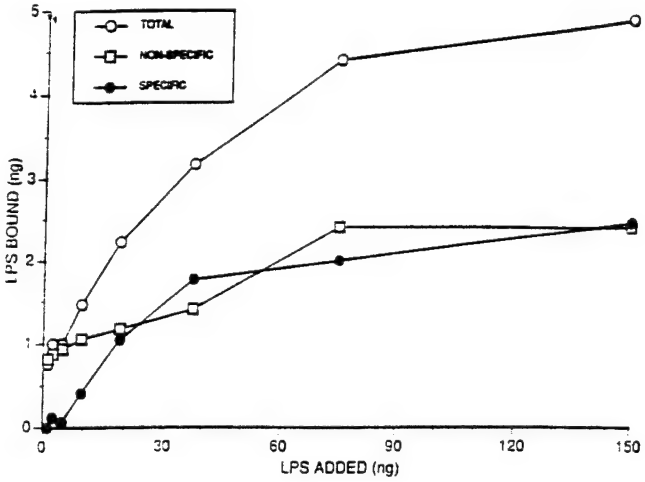

FIG. 1. **Binding of LPS to immobilized Hb.** $\alpha\alpha$Hb (1 μg/well) was immobilized in microtiter plate wells, and ^{125}I-LPS was added. Bound LPS was determined by gamma counting, and specific binding was calculated by subtracting bound ^{125}I-LPS in wells without Hb.

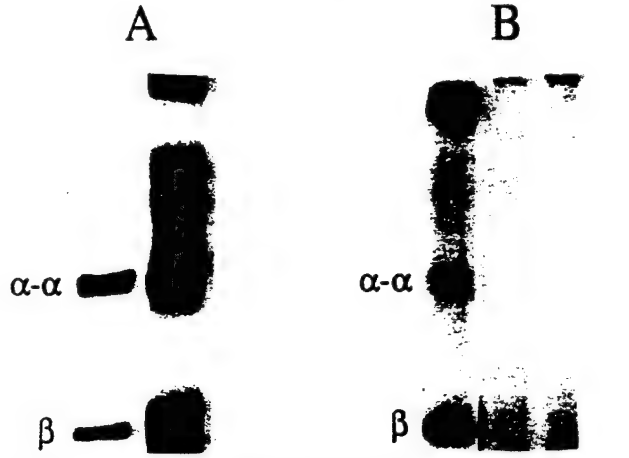

FIG. 2. **Photoaffinity labeling of Hb with ^{125}I-LPS-ASD.** ^{125}I-LPS-ASD was incubated with $\alpha\alpha$Hb, photolyzed with UV light, and electrophoresed in SDS and 2-mercaptoethanol. Following electrophoresis, the gel was stained with Coomassie Blue (*A, left lane*), dried, and subjected to autoradiography (*A, right lane*). Another photoaffinity-labeled $\alpha\alpha$Hb preparation from a separate experiment is shown (*B, left lane*), along with controls that consisted of an incubation mixture containing 100-fold excess unlabeled LPS as a blocking agent to demonstrate inhibition of specific binding (*B, middle lane*) and ^{125}I-LPS-ASD alone (*B, right lane*).

ing occurred with a calculated K_d of 4.7×10^{-4} g/liter (3.1×10^{-8} M, assuming a monomer molecular mass of 1.5×10^4 for *E. coli* LPS).

LPS Photoaffinity Labeling of Hb—In the presence of SDS, $\alpha\alpha$Hb electrophoresed as two bands, an-$\alpha\alpha$ cross-linked dimer of 33 kDa and monomer β chains of 16.5 kDa (Fig. 2*A, left lane*). Autoradiography of Coomassie Blue-stained gels reproducibly demonstrated binding of the photoaffinity LPS probe to both chains of $\alpha\alpha$Hb (Fig. 2*A, right lane* and 2*B, left lane*). Binding of the LPS photoaffinity probe to Hb was totally blocked by 100-fold excess unlabeled LPS (Fig. 2*B, middle lane*) indicating that the binding was specific. Although the LPS photoaffinity probe to Hb labeled both types of Hb chain, there was substantially more labeling of the β chains. When the monomer and dimer bands were excised from the gel and the associated counts/min were determined by gamma counting, 72% of counts/min were in the β band (range, 57–80%), and 28% were in the $\alpha\alpha$ dimer band (range, 20–43%) in three independent experiments. Binding of the LPS photoaffinity probe to native HbA_0 also was demonstrated (data not shown).

Ultrafiltration of Hb and LPS—87–89% of the *E. coli* LPS in Hb/LPS mixtures was filtered through a 300-kDa membrane (although in aqueous solutions, highly aggregated LPS typically has a molecular weight greater than 10^6), whereas only 10.2% of *E. coli* LPS alone was filterable (Table I). Similarly, 91–97% of the *P. mirabilis* LPS in Hb/LPS mixtures was filtered through the 300-kDa cut-off membrane, whereas only 15.6% of *P. mirabilis* LPS alone was filterable (Table I). This increase in LPS filterability was also demonstrated using radiolabeled LPS (^{125}I-labeled *P. mirabilis* LPS); only 22% of the LPS alone was filterable, but 78% of LPS in the presence of $\alpha\alpha$Hb was filterable (data not shown). 64–72% of the *P. mirabilis* S1959 LPS was filtered through a 100-kDa membrane in the presence of Hb, but LPS alone was not filterable (Table I). Approximately 90% of the total Hb protein in each of the three Hb/LPS mixtures, and from filtrates of Hb alone, was detected in filtrates of the 300- and 100-kDa membranes (data not shown).

Sucrose Centrifugation of LPS and Hb—High speed ultracentrifugation (52,000 × *g* for 4 h) of Hb alone demonstrated sedimentation of Hb part way into a 4–20% sucrose gradient. Whereas LPS alone sedimented to the bottom of the gradient, most of the LPS (80–95%) in Hb/LPS mixtures had a sedimentation rate similar to that of Hb (Fig. 3). In four independent experiments, Hb/LPS mixtures demonstrated co-migration of both components (Hb and LPS). Slow speed centrifugation (2,900 × *g* for 30 min) through 5% sucrose also demonstrated co-migration of Hb and LPS. 70.2% of LPS sedimented into the bottom fraction in the absence of protein, whereas only 4–11% sedimented in the presence of any of the three Hb preparations (Fig. 4). Conversely, only 10.7% of LPS alone remained above the sucrose layer, whereas, in the presence of Hb, 65–80% of LPS remained in the top layer. A similar redistribution of LPS from the bottom fraction (in the absence of protein) to the top fraction was observed in the presence of HSA. No detectable Hb or HSA entered the sucrose layer in either the absence or presence of LPS. Redistribution of LPS into the top layer was a saturable process, and binding of LPS to Hb occurred with a calculated K_d of 6.3×10^{-4} g/liter (6.3×10^{-8} M assuming a monomer molecular mass of approximately 10,000 Da for *S. typhimurium* Rc LPS) (data not shown). This number is in close agreement with the K_d calculated from the microtiter plate binding assay (4.7×10^{-4} g/liter) described above.

Nondenaturing Gel Electrophoresis of LPS and Hb—100% of ^{14}C-labeled *S. typhimurium* LPS alone remained within the first 9 mm of the gel (gel pieces 1–3), whereas 43% of the LPS

TABLE I
Ultrafiltration of E. coli O26:B6 and P. mirabilis S1959 LPS, Hb, and Hb/LPS mixtures

Each experiment was performed three times and the mean ± S.D. is shown. Percent of LPS filtered was determined with the chromogenic LAL test. LPS was quantified with reference to standard curves consisting of the respective LPS/protein mixture prior to filtration (see "Experimental Procedures").

	E. coli LPS filtered (300-kDa[a] filter)	*P. mirabilis* LPS filtered	
		100-kDa[a] filter	300-kDa[a] filter
	%	%	
LPS alone	10.2 ± 2.3	0	15.6 ± 5.6
$\alpha\alpha$Hb alone	0[b]	0	0
$\alpha\alpha$Hb + LPS	87.3 ± 8.0	63.6 ± 18.7	97.1 ± 1.5
$\alpha\alpha$HbCO alone	0	0	0
$\alpha\alpha$HbCO + LPS	89.3 ± 1.5	71.1 ± 4.0	90.9 ± 4.5
HbA_0 alone	0	0	0
HbA_0 + LPS	88.1 ± 3.7	71.6 ± 8.8	93.5 ± 8.6

[a] Molecular mass cut-off of the filter.
[b] Lack of detectable LPS indicates that the starting preparations of Hb were endotoxin-free.

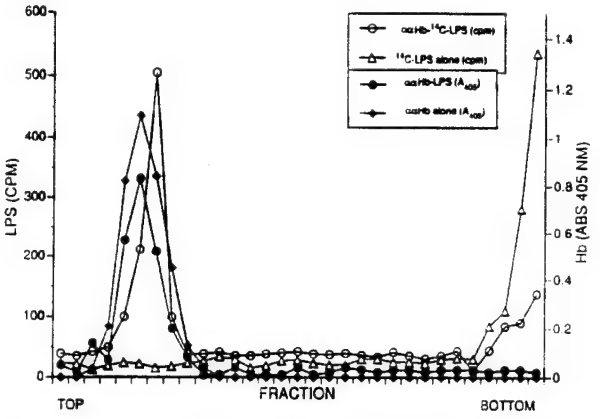

FIG. 3. **Sucrose density centrifugation of Hb/LPS.** ^{14}C-LPS was incubated with $\alpha\alpha$Hb (100 mg/ml), and the mixture was centrifuged through a 4–20% continuous sucrose gradient. 0.4-ml fractions were assayed for hemoglobin by absorbance at 405 nm (*closed symbols*) and for LPS by scintillation counting (*open symbols*).

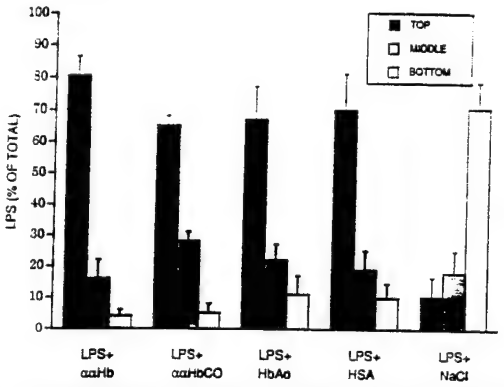

FIG. 4. **Centrifugation of LPS and Hb through 5% sucrose.** ^{14}C-Labeled LPS was incubated with $\alpha\alpha$Hb, $\alpha\alpha$HbCO, HbA_0, or HSA. These LPS/protein mixtures, and LPS in NaCl, were centrifuged through a layer of 5% sucrose as described under "Materials and Methods." ^{14}C-Labeled LPS was measured by scintillation counting in the top (*solid columns*), middle (*stippled columns*), and bottom (*open columns*) zones of the centrifuged samples. Results are expressed as the mean ± S.D. of four independent experiments.

in the $\alpha\alpha$Hb/LPS mixture migrated farther into the gel and co-migrated with $\alpha\alpha$Hb (gel pieces 6–17) (Fig. 5). Recovery of the total applied counts/min from all the gel pieces was 93%. A second electrophoresis experiment with ^{14}C-labeled *S. typhimurium* LPS demonstrated that 45% of the LPS co-migrated with the $\alpha\alpha$Hb (data not shown), and an identical experiment performed with ^{125}I-labeled *E. coli* LPS demonstrated 23% co-migration (data not shown).

Precipitation of LPS and Hb by Ethanol—At each of the temperatures studied (4, 20, or 37 °C) and both in the presence and absence of Hb, 90–100% of *E. coli* O26 LPS or *P. mirabilis* S1959 LPS was precipitated by ethanol (data not shown). Precipitation of Hb alone was variable (13.9–37.5%). However, in almost all conditions studied, the presence of LPS increased the amount of precipitated Hb protein (Table II). Mixtures of *E. coli* LPS with each of the Hb solutions, incubated and precipitated at each of the three temperatures, demonstrated from 14.1 to 42.5% more Hb precipitated than when Hb was precipitated in the absence of LPS. Similarly, mixtures of *P. mirabilis* S1959 LPS and each of the Hb preparations, incubated and precipitated at 20 and 37 °C, demonstrated increased Hb precipitation (from 6.7 to 12.6% more protein than Hb alone) (Table II). At 4 °C, the HbA_0/*P. mirabilis* LPS mixture demonstrated increased precipitation of HbA_0 (22.5%) compared with HbA_0 precipitation in the absence of LPS. In contrast, the precipitation

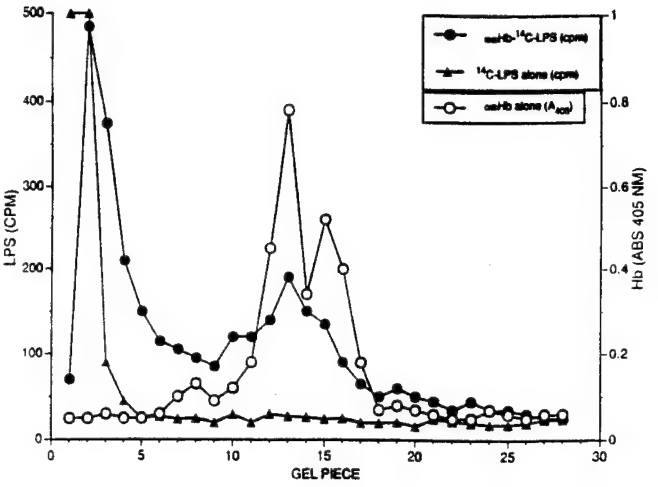

FIG. 5. **Electrophoresis of LPS and Hb.** ^{14}C-Labeled LPS was incubated with $\alpha\alpha$Hb, and the $\alpha\alpha$Hb/LPS mixture or LPS alone was electrophoresed in polyacrylamide in the absence of SDS, as described under "Materials and Methods." ^{14}C-Labeled LPS was measured by scintillation counting of gel pieces (*closed symbols*), and $\alpha\alpha$Hb was monitored by absorbance at 405 nm (*open circles*).

TABLE II

Percent of cell-free hemoglobin precipitated by ethanol in the absence and presence of P. mirabilis S1959 or E. coli O26:B6 endotoxins (LPS)

The mean value of eight replicate precipitations ± S.D. is shown. Percent of Hb precipitated was determined by measurement of protein.

Type of Hb	Temperature[a]	Hb alone	Hb + LPS (*P. mirabilis*)	Hb + LPS (*E. coli* O26)
	°C[b]			
$\alpha\alpha$Hb	4	37.5 ± 2.6	38.3 ± 0.8 (0.8)[b]	67.3 ± 1.9 (29.8)
$\alpha\alpha$Hb	20	26.8 ± 5.9	35.9 ± 10.8 (9.1)	69.3 ± 2.5 (42.5)
$\alpha\alpha$Hb	37	22.7 ± 8.7	32.1 ± 4.9 (9.4)	56.9 ± 6.5 (34.2)
$\alpha\alpha$HbCO	4	34.1 ± 1.9	35.4 ± 0.8 (1.3)	69.7 ± 1.9 (35.6)
$\alpha\alpha$HbCO	20	25.4 ± 5.8	33.3 ± 7.9 (7.9)	66.8 ± 17.2 (41.4)
$\alpha\alpha$HbCO	37	26.2 ± 6.9	32.9 ± 5.6 (6.7)	59.5 ± 6.5 (33.3)
HbA_0	4	17.5 ± 0.6	40.0 ± 1.0 (22.5)	31.6 ± 0.6 (14.1)
HbA_0	20	13.9 ± 5.8	26.5 ± 13.5 (12.6)	45.9 ± 6.2 (32.0)
HbA_0	37	20.3 ± 10.1	31.9 ± 1.9 (11.6)	44.5 ± 4.4 (24.2)

[a] Temperature of incubation and precipitation.

[b] Numbers in parentheses indicate increase in percent of Hb precipitated by ethanol (67%, final concentration) in the presence of endotoxin.

of $\alpha\alpha$Hb or $\alpha\alpha$HbCO was not significantly altered by *P. mirabilis* LPS at 4 °C. *E. coli* O26:B6 LPS co-precipitated significantly more of each Hb than *P. mirabilis* LPS at all conditions tested, except for the incubation of HbA_0 with LPS at 4 °C (Table II).

LAL Reactivity of Hb and LPS Mixtures—Using the chromogenic LAL test with *Limulus* lysate, which had been diluted 20-fold in order to expand the measurable range of LPS concentrations, *E. coli* O26:B6 LPS was assayed alone and in the presence of Hb or HSA (Fig. 6*A*). All three Hb preparations (and HSA) resulted in enhanced activation of LAL over a wide range of LPS concentrations. Sensitivity of LAL for low concentrations of LPS was increased approximately 10-fold in the presence of proteins. Comparable enhancement of the biological activity of LPS in the LAL test by each of the three Hb preparations and by HSA also was demonstrated using *P. mirabilis* S1959 LPS (Fig. 6*B*). Similar enhancement effects were ob-

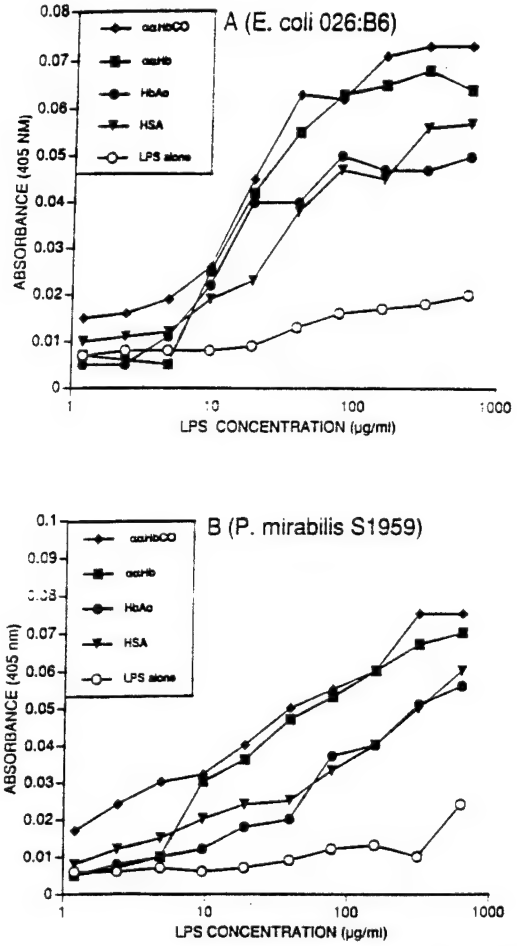

FIG. 6. **Enhancement of LPS activation of *Limulus* amebocyte lysate by Hb.** Dilutions of *E. coli* O26:B6 LPS (*A*) or *P. mirabilis* S1959 LPS (*B*) in ααHbCO (◆), ααHb (■), HbA_o (●), HSA (▼) or NaCl (○), were assayed with the chromogenic LAL test. Absorbances at 405 nm were measured at 5 min. All protein concentrations were 1 mg/ml. Incubations were performed in triplicate, and the mean is shown.

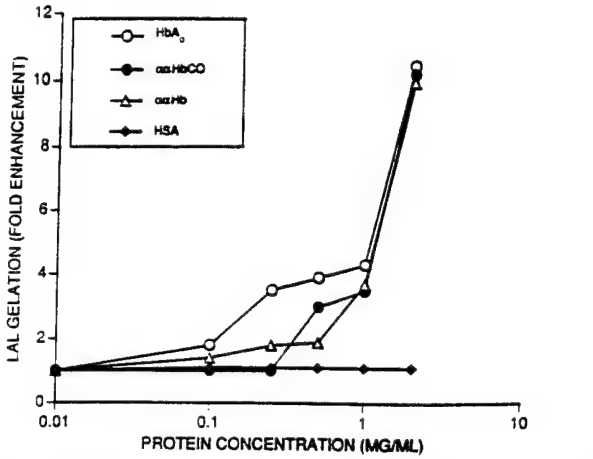

FIG. 7. **Enhancement of LPS activation of *Limulus* amebocyte lysate by Hb.** Mixtures of LPS (100 pg/ml *E. coli* O26:B6) and HbA_o (○), ααHbCO (●), ααHb (△), or HSA (◆) were assayed with LAL, using gelation as the end point. Protein concentrations ranged from 0.01 to 2 mg/ml. Enhancement of activation of LAL was calculated by comparison of the gelation time of each mixture with the gelation times for LPS solutions in 0.9% NaCl. 100 pg/ml LPS in 0.9% NaCl solution gelled *Limulus* amebocyte lysate in 2.5 h. Similar results were obtained from each of three independent experiments.

tained from three independent experiments. The enhancement by each Hb of LAL activation also was demonstrated with the gelation LAL test, using undiluted *Limulus* lysate for maximum LPS sensitivity, and enhancement was shown to be dependent on protein concentration (Fig. 7). Prominent enhancement (2–10-fold) was observed over a log range of concentrations for each Hb (from 0.2 to 2 mg/ml Hb). At 100 μg/ml Hb, less than 2-fold enhancement was demonstrated with ααHb and HbA_o; ααHbCO at 100 μg/ml did not demonstrate enhancement. Three independent experiments failed to demonstrate enhancement by any tested concentration of HSA (from 0.01 to 2 mg/ml) in the gelation LAL test; this is in contrast to the reproducible enhancement by HSA demonstrated utilizing diluted LAL in the chromogenic test.

DISCUSSION

ααHb is a cell-free preparation of a derivatized human hemoglobin (cross-linked between α chains), which has adequate oxygen carrying and releasing properties and also an acceptable *in vivo* half-life (4–24 h in various animals) (1, 26–29). Despite these favorable characteristics, *in vivo* animal studies with ααHb and other purified cross-linked hemoglobin preparations have demonstrated significant toxicity (6, 7, 30, 31), thus limiting their potential clinical use. However, it is uncertain whether ααHb is intrinsically toxic, or if the previously described *in vivo* toxicity has primarily resulted from associated endotoxin or contaminating stromal phospholipids (6). Toxicity due to associated LPS has been described previously (6, 9, 10), and evidence for synergistic toxicity of LPS and Hb also has been reported (10, 32). Due to the large volumes of Hb that would be infused into a patient during resuscitation, LPS contamination of Hb would be a potentially major limitation to the clinical use of solutions of Hb.

Preliminary experiments in our laboratory suggested that formation of ααHb·LPS complexes could contribute to the observed *in vivo* toxicity of preparations of Hb by modification of the physical structure and biological activity of contaminating LPS. Therefore, we performed experiments to determine if physical interactions between Hb and LPS were demonstrable and if such interactions altered the biological activity of LPS. Six different techniques provided evidence to support the conclusion that Hb and LPS formed stable complexes. Firstly, LPS bound to immobilized Hb in a saturable manner. The calculated K_d (4.7×10^{-4} g/liter based on the microtiter plate binding assay, and 6.3×10^{-4} g/liter based on the sucrose centrifugation assay) indicated that the interaction is of moderate affinity. When expressed as molar values, these binding constants of LPS for Hb were in the same range as binding constants reported for other LPS binding proteins (33–35). Second, specific binding of the LPS photoaffinity probe ^{125}I-LPS-ASD to Hb confirmed complex formation, and demonstrated that binding of LPS to the β chains of Hb is particularly prominent. Third, a portion of the LPS in the presence of Hb demonstrated increased electrophoretic mobility, and co-electrophoresed with Hb. This result is consistent with a process of LPS disaggregation and Hb·LPS complex formation. Fourth, in the presence of Hb (ααHb, ααHbCO, or HbA_o) the majorities (approximately 70–90%) of both LPSs utilized (*E. coli* O26:B6 or *P. mirabilis* S1959) were detectable in filtrates of 300- and 100-kDa ultrafiltration membranes, in contrast to the lack of filterability of LPS in the absence of Hb. The filterability of LPS in the presence of Hb was consistent with the presence of relatively low molecular weight Hb·LPS complexes. This is in contrast to the high molecular weight aggregation state of LPS alone (typically $>10^6$). Fifth, the reduction in sedimentation velocity, determined by sucrose centrifugation of each LPS after incubation with each of the three Hb preparations, further supported the conclusion that Hb altered the physical properties of LPS, and the co-migration of LPS and Hb provided additional evidence that Hb

and LPS formed stable complexes. Finally, the co-precipitation by ethanol of each of the three Hb preparations with each LPS provided evidence that LPS altered the physical properties of Hb and also was consistent with the presence of stable Hb·LPS complexes. Hb/LPS ratios differed considerably for performance of experiments using these models because of the variable methods required for Hb and LPS detection, yet each of these models supported the conclusion that complex formation had occurred.

Formation of Hb·LPS complexes was demonstrated for native HbA_o as well as for the derivatized cross-linked $\alpha\alpha$Hb. This finding provided further evidence for the general conclusion that $\alpha\alpha$Hb is similar physiologically to native hemoglobin (29). Additionally, this observation suggests that native hemoglobin, released into the circulation by *in vivo* hemolysis of erythrocytes (*e.g.* as can be observed during Gram-negative bacterial sepsis), may interact with circulating endotoxins.

Interaction of LPS with cross-linked and native Hb preparations also was associated with increased biological activity (enhanced LAL activation) of LPS. In the presence of all three Hb preparations, LPS activated LAL both more rapidly and at lower concentrations than in the absence of Hb. These results are in agreement with the previous preliminary finding (36) that preparations of $\alpha\alpha$Hb (ranging from 0.001–100 mg/ml) enhanced the ability of *E. coli* lipopolysaccharide (O55:B5) to activate LAL. Confirmation of increased biological activity of LPS, in the presence of Hb, also has been provided by the observations that generation of mononuclear cell tissue factor by LPS is enhanced by Hb (37) and that generation of tissue factor from endothelial cells is enhanced by Hb (38). Lipid A was capable of interacting with Hb to produce an increase in LPS biological activity, although the -fold enhancement of lipid A activity by Hb was less than that observed with complete LPS.[1] Hb·LPS complex formation and LAL enhancement by $\alpha\alpha$HbCO, results which were similar to those observed with $\alpha\alpha$Hb and HbA_o, established that these properties of Hb do not involve methemoglobin production and are not related to the state of oxygenation of Hb.

In contrast to the reproducible enhancement effect of Hb, our finding that enhancement of LPS biological activity by HSA was observed with one of our assay conditions (the diluted LAL chromogenic assay) but not with another (the undiluted gelation assay) suggests that the Hb-LPS and Hb-HSA interactions may not be similar. Variable effects of HSA on LPS biological activity have been described; the biological activity of LPS in the presence of HSA previously has been shown to be increased (39), decreased (40), or both increased and decreased, depending on HSA concentration (41).

The increased reactivity of LPS, when complexed with Hb, the associated decrease in molecular weight and density of LPS, and the altered electrophoretic mobility of LPS may be explained by a detergent-like effect of Hb on LPS; *i.e.* Hb may reduce the size of the LPS aggregates, making LPS more soluble and more biologically available for activation of the LAL enzymatic cascade. Disaggregation of LPS is a well recognized phenomenon in plasma (42, 43), although this process previously has been demonstrated to result in detoxification of LPS (43). In contrast to the effect of Hb, other proteins that bind LPS and result in altered LPS biological activity, such as melittin (44), bacterial outer membrane 39-kDa protein (45), lysozyme (33), complement proteins (46), bactericidal/permeability-increasing protein (47), or polymyxin B (48), cause a decrease in LPS toxicity. Although the mechanism of Hb enhancement of LPS biological activity is likely to involve LPS disaggregation, it is also possible that LPS undergoes a chemical modification in the presence of Hb, perhaps similar to the process of phospholipid peroxidation that has been demonstrated when oxyhemoglobin binds phospholipid (49).

The increased biological activity (*e.g.* in LAL activation) of LPS in the presence of $\alpha\alpha$Hb and the other Hb preparations is of potential physiological significance since $\alpha\alpha$Hb would likely be infused into trauma patients with concomitant endotoxemia. In rabbits, LPS and $\alpha\alpha$Hb have been shown to have synergistic toxicity (10), and, in dogs, impure hemoglobin, produced by hemolysis of red blood cells, was shown to enhance the toxicity of infused LPS (32). Importantly, the deleterious effects of interactions of LPS with blood cells (*e.g.* activation of mononuclear cells) and the endothelium (*e.g.* induction of a procoagulant state) might be augmented in the presence of $\alpha\alpha$Hb. A patient requiring $\alpha\alpha$Hb for resuscitation would potentially require a plasma concentration of 50 mg/ml (5 g/dl) of $\alpha\alpha$Hb in order to provide adequate oxygen carrying capacity. This is a concentration 25-fold greater than that demonstrated in Fig. 7 to enhance LAL gelation 10-fold. Therefore, enhancement of LPS biological activity by $\alpha\alpha$Hb would constitute a serious clinical risk if the patient was endotoxemic. Accordingly, successful development and clinical utilization of $\alpha\alpha$Hb as a red blood cell substitute will depend on a more complete understanding of the interaction between $\alpha\alpha$Hb and LPS.

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RESEARCH

Human hemoglobin increases the biological activity of bacterial lipopolysaccharides in activation of *Limulus* amebocyte lysate and stimulation of tissue factor production by endothelial cells in vitro

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SUMMARY. **Previous studies have demonstrated that hemoglobin (Hb) and bacterial endotoxin (lipopolysaccharide, LPS) form stable complexes and result in disaggregation of macromolecular LPS. To examine the effect of complex formation on LPS biological activity, we investigated the ability of Hb to alter LPS-induced activation of the coagulation cascade of *Limulus* amebocyte lysate (LAL) and expression of tissue factor from human endothelial cells. Both native HbA_0 and derivatized (covalently cross-linked) hemoglobin resulted in prominent enhancement of LAL activation and endothelial cell tissue factor production by *Proteus mirabilis* LPS. No substantial differences were observed between the enhancement effect of Hb on *P. mirabilis* smooth and rough LPS, indicating a dominant role for the lipid A component of LPS. Rough (Re) *Salmonella minnesota* 595 LPS also demonstrated both enhanced activation of LAL and stimulation of endothelial cell tissue factor in the presence of Hb. In contrast, neither lipid A nor singly dephosphorylated or partially deacylated Re LPS manifested significant enhancement of LAL activation by Hb, and partially deacylated Re LPS showed no enhancement of endothelial cell tissue factor by Hb. These results suggest that the Kdo moieties, as well as the phosphate residues and fatty acyl moieties of lipid A, may be involved in the interaction of Hb with LPS. Comparison of Hb with other endotoxin binding proteins for ability to cause enhancement of LPS biological activity demonstrated more prominent enhancement with lipopolysaccharide binding protein (LBP) than that observed with Hb, lesser enhancement with albumin, and no enhancement effect with IgG or transferrin.**

The ability of hemoglobin (Hb) solutions, in the absence of erythrocytes, to act as oxygen carriers has been recognized for several decades.[1] Recently, highly purified human Hb preparations have been produced that demonstrate adequate oxygen binding and releasing properties,[2–4] and are being developed for potential use as an oxygen-transporting resuscitation fluid.[5,6] These materials have been rigorously purified of red cell stromal (lipid) contaminants, and in some instances have been covalently cross-linked between chains of the Hb tetramer to maintain the tetrameric structure outside of the erythrocyte and prolong intravascular persistence.[7] However, Hb, removed from the erythrocytic milieu, has been associated with many toxic effects when administered in vivo, prominent among which are fever, hypertension, renal failure, hepatic necrosis and coagulopathy.[8–11] Several potential causes of these toxicities have been described, including the association of multisystem dysfunction with contamination of Hb by residual erythrocyte phospholipids and/or bacterial endotoxin lipopolysaccharide (LPS).[8,12–14]

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Recently, we have shown that complex formation occurs between Hb and LPS, resulting in a significant decrease in the aggregate molecular weight of LPS, and postulated that Hb-LPS complex formation was partially responsible for the observed toxicities of in vivo infusions of Hb.[15] In addition, in preliminary experiments the biological activity of LPS was increased, as demonstrated by enhanced LPS activation of *Limulus* amebocyte lysate (LAL) in the presence of Hb.[15] The formation of Hb-LPS complexes also resulted in enhanced LPS activation of human peripheral blood mononuclear cells.[16] We have hypothesized that the binding and disaggregation of LPS by Hb increases LPS solubility, resulting in augmented biological activity. However, the mechanism and molecular constituents of LPS required for this effect have not been identified. The present study was designed to evaluate the requirements for different components of LPS in producing the enhancement effect of Hb.

Proteus mirabilis LPS, differing in polysaccharide composition, were studied because they provided a series of probes for evaluation of the role of the O chain polysaccharides in the enhancement process. Rough (Re) *Salmonella minnesota* 595 LPS and selected chemical derivatives were used to evaluate the contribution of fine structural components of the core and lipid A moieties. Hb preparations studied have included cross-linked human Hb (αα Hb) because this Hb preparation is being developed as a red blood cell substitute, and native, unmodified HbA_0 to ensure that we were studying an intrinsic property of Hb. We have also investigated carbonmonoxyhemoglobin (αα HbCO) as a form of Hb that would have a greatly reduced propensity to generate methemoglobin. Our data have demonstrated that the hydrophobic components of LPS, i.e. lipid A and the deep rough portion of the core region of LPS, are responsible for the interactions with human Hb that lead to the enhancement of LPS activity in the LAL assay and stimulation of the production of tissue factor by endothelial cells.

MATERIALS AND METHODS

Reagents

Sterile, endotoxin-free water and 0.9% NaCl were purchased from Travenol Laboratories (Deerfield, IL, USA). RNAse and DNAse were purchased from Sigma Chemical Co. (St Louis, MO, USA). Human serum albumin (HSA) (25%, for injection) was purchased from Nybcen (New York, NY, USA), human immunoglobulin (IgG) (185 mg/ml) from Armour Pharmaceutical Co. (Kankakee, IL, USA) and human transferrin from Calbiochem (La Jolla, CA, USA).

Glassware

All glassware was rendered endotoxin-free by heating at 190°C in a dry oven for 4 h.

Hemoglobin

Human Hb was prepared and purified, as described previously,[2] by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR), San Francisco, CA, USA. Human Hb was covalently cross-linked between α chains with bis(3,5-dibromosalicyl)fumarate (αα Hb). The αα Hb stock solution was 9.6 g/dl, pH 7.4 in Ringers acetate, and contained less than 0.4 EU/ml endotoxin (referenced to *Escherichia coli* lipopolysaccharide B, O55:B5, Difco Laboratories, Detroit, MI, USA), as determined by LAL.[17] The αα Hb stock solution was stored at –70°C, and diluted with sterile, pyrogen-free 0.9% NaCl prior to use. αα HbCO (95% HbCO), produced by incubation of the αα Hb solution with CO, also was at 9.6 g/dl, pH 7.4. Purified non-crosslinked A_0 (HbA_0), 8.4 g/dl, was prepared from Hb by ion exchange HPLC, as described previously.[3]

Lipopolysaccharide binding protein

Purified rabbit LPS binding protein (LBP) was the generous gift of Dr Peter Tobias, The Scripps Research Institute, La Jolla, CA, USA.

Bacterial LPS

Proteus LPS

P. mirabilis S 1959 smooth LPS, Ra type (R110) and Re type (R45) rough mutants of S1959, and *P. mirabilis* O3 smooth LPS were prepared at the Institute of Microbiology and Immunology, University of Lodz, Poland. *P. mirabilis* R45 LPS contains only lipid A, two residues of 2-keto-3-deoxyoctulosonic acid (Kdo) and two 4-amino-arabinose (Ara4N) residues.[18,19] *P. mirabilis* R110 LPS contains the complete core oligosaccharide linked to lipid A.[20] Smooth LPS were extracted with phenol-water according to the Westphal method,[21] whereas rough LPS were extracted by the phenol-chloroform-petroleum ether method (PCP) according to Galanos.[22] Crude *Proteus* LPS were further purified by sequential treatment with RNAse and DNAse, followed by ultracentrifugation at 100,000 x g for 3 h, as described previously.[23]

Salmonella LPS

The deep rough *S. minnesota* R 595 LPS was extracted by the PCP method[22] and then, in order to study the role of the hydrophobic part of endotoxin (lipid A) on LAL activation, a series of partially chemically degraded derivatives were prepared as follows (see Table for LPS compositions).

Lipid A

S. minnesota R 595 lipid A was prepared from 134 mg of R 595 LPS by hydrolysis of the Kdo and Ara4N

residues with sodium acetate (pH 4.4) for 1 h at 100°C.[24] The hydrolysate was dialysed to obtain purified lipid A (67% yield) and then lyophilized and stored at 4°C.

Monophosphoryl lipid A

Monophosphoryl lipid A (MPL) was obtained from 200 mg of R 595 LPS by hydrolysis with 0.1 N HCl for 45 min at 100°C, conditions known to remove the phosphate residue from the reducing glucosamine,[25] and the Kdo and Ara4N residues from non-reducing glucosamine. The MPL hydrolysate was centrifuged at 10,000 x g for 30 min, washed twice with water, and then lyophilized (58% yield).

Singly deacylated LPS (OH37 LPS)

OH37 LPS was produced from 380 mg of R 595 LPS by hydrolysis of a single ester-bound 3-hydroxy-tetradecanoyl fatty acid from the reducing glucosamine of LPS with 0.2 N NaOH for 30 min at 37°C; 93% removal of this fatty acid has been demonstrated with this procedure.[25] The hydrolysate was then cooled to 4°C and neutralized to pH 6.5 with 0.1 N HCl. Released fatty acids were extracted with $CHCl_3$/MeOH (2:1) followed by precipitation of OH37 LPS with EtOH/acetone (2:1) at 0°C. The OH37 LPS sediment was centrifuged at 10,000 x g for 30 min, washed twice with cold EtOH, resuspended in water, and lyophilized (61% yield).

Multiply deacylated LPS (OH56 LPS)

OH56 LPS was produced from 190 mg of OH37 LPS by hydrolysis in 0.2 N NaOH for 60 min at 56°C.[25] The hydrolysate was cooled to 4°C, and then neutralized to pH 6.5 with 0.1 N HCl, precipitated by EtOH/acetone (2:1) at 4°C, and centrifuged at 10,000 x g for 30 min. The precipitate was washed twice with cold EtOH, resuspended in water and lyophilized (50% yield).

Salmonella abortus equi

S. abortus equi LPS was the generous gift of Dr Chris Galanos, Max-Planck Institute, Freiburg, Germany.

E. coli LPS

E. coli O26:B6 (Westphal preparation obtained by hot phenol-water extraction[21] was purchased from Difco Laboratories (Detroit, MI, USA). This crude *E. coli* LPS was further purified as described above for *Proteus* LPS.[23] For some experiments, purified *E. coli* O26 LPS then was electrodialysed at 0.47 mA for 8 h at 4°C and aliquots were neutralized with triethylamine, 0.01 N NaOH or 0.1 M $CaCl_2$ to form the triethylamine, sodium and calcium salts,[26] respectively. *E. coli* F515 LPS, prepared from the rough strain *E. coli* F515 (Re), was the generous gift of Dr Chris Galanos, Max-Planck Institute, Freiburg, Germany.

Rhodobacter LPS

Non-enterobacterial *Rhodobacter spheroides* ATCC 17023, *Rhodobacter capsulatus* 37b4 and *Rhodopseudomonas viridis* LPS were kindly provided by Dr Hubert Mayer, Max-Planck Institute, Freiburg, Germany.

Chemical analysis

Colorimetric methods were used to determine the con-

Table. Chemical composition of *S. minnesota* 595 LPS and its chemically degraded derivatives

Components (nmol/mg LPS)*		LPS				
		595	OH37	OH56	MPL	Lipid A
Kdo		655	825	811	6	53
$PO(OH)_2$		1160	1733	749	897	1052
C_{12}	Total	401†	598	76†	1137	530
	Ester	439	472	87	500	447
C_{14}	Total	214	147	0†	471	529
	Ester	76	100	14	119	167
C_{14}-OH	Total	1415	1278	703	2146	1623
	Ester	650	22	0	1092	812
C_{16}	Total	205	163	54†	214	154†
	Ester	155	155	63	117	182

*2-keto-3-deoxy-octulosonic acid (Kdo) and phosphate ($PO(OH)_2$) residues were determined in hydrolysates of LPS utilizing colorimetric reactions with thiobarbituric acid and molybdate complexes, respectively; fatty acids (C_{12}, dodecanoic acid; C_{14}, tetradecanoic acid, C_{14}-OH, 3-hydroxy-tetradecanoic acid, and C_{16}, hexadecanoic acid) were determined as their methyl esters, in basic (ester bound) or acid (total = ester and amide bound) hydrolysates of LPS.

†Determination of total fatty acids is slightly underestimated due to chemical degradation during hydrolysis.

Abbreviations: 595, untreated *S. minnesota* 595 LPS (Mr 2867‡); OH37, singly deacylated 595 LPS (Mr 2641); OH56, multiply deacylated 595 LPS (Mr 1785); MPL, monophosphoryl lipid A (partially dephosphorylated 595 LPS) (Mr 2094); lipid A, product after acetic acid hydrolysis of LPS 595 (Mr 2298).

‡Monomer molecular mass (Mr) of each LPS was estimated, assuming complete substitution of each structural component.

tent of Kdo[24] and phosphate residues;[27] gas-liquid chromatography (GLC) was performed for analysis of methyl esters of fatty acids.[28]

Turbidity measurements

0.18 ml of LPS solutions (1 mg/ml in 0.9% NaCl), in the absence or presence of ααHb (0.01–1 mg/ml) was incubated for 90 min at 37°C, and absorbance at 620 nm was determined, as a measurement of turbidity, in a temperature-controlled plate reader (Kinetic-QCL, Whittaker Bioproducts Inc., Walkersville, MD, USA).

Limulus amebocyte lysate

Amebocyte lysates were prepared from *Limulus polyphemus* (the North American horseshoe crab) by lysis of washed amebocytes in distilled water, as described previously.[17,29] Limuli were obtained from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA, USA.

Chromogenic substrate

Chromogenic substrate S-2423 (AB Kabi Vitrum,

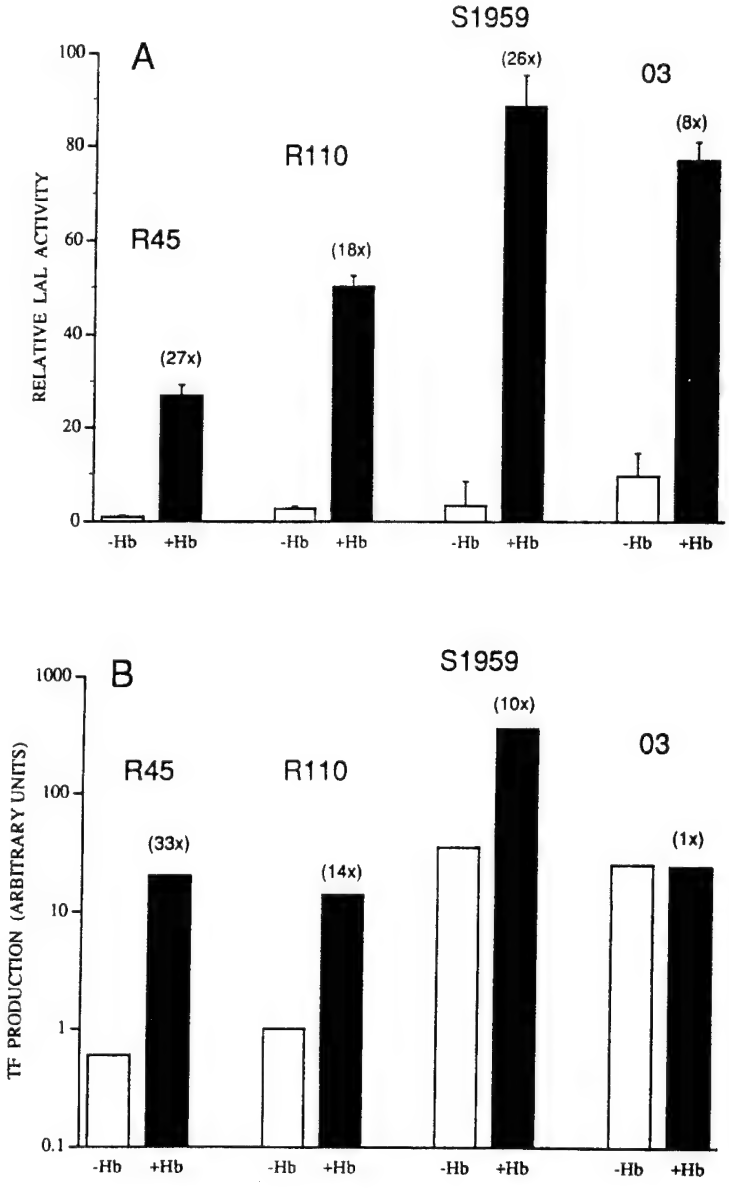

Fig. 1 — Enhancement by Hb of the activation of LAL and production of endothelial cell tissue factor by *Proteus* LPS. **(A)** LAL reactivities of LPS (500 ng/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (1 mg/ml) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of *P. mirabilis* R45 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R45 LPS concentration. 500 ng/ml R45 LPS was assigned a relative LAL activity of 1. The fold increase in LAL activity of each LPS, induced by Hb, is indicated in parentheses. Samples were assayed with 8 replicates, and results are expressed as the mean ± 1 SD. **(B)** Cultured human umbilical vein endothelial cells were incubated with *Proteus* LPS (10 μg/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (10 mg/ml). TF activities then were determined with a plasma recalcification assay. The fold increase in TF induced by Hb for each LPS is indicated in parentheses. The means of 4 wells are presented.

Molndal, Sweden), utilized in the chromogenic LAL test (below) to measure activation of the LAL proteolytic cascade, was the gift of Dr Petter Friberger, and was reconstituted with pyrogen-free water.

Chromogenic LAL test

Activation of LAL by LPS was quantified with a chromogenic LAL test, as described previously.[15] For most experiments, the LAL was freshly diluted 1:20 in 0.9% NaCl prior to use. For experiments utilizing LBP, undiluted rather than 1:20 LAL was used in order to increase sensitivity to the effects of LPS and LBP.

Endothelial cell tissue factor assay

Production of tissue factor (TF) procoagulant activity from human umbilical vein endothelial cells (obtained from Clonetics Corp., San Diego, CA, USA) was determined as described previously.[30] Briefly, confluent human endothelial cell monolayers in 96-well tissue culture plates were incubated with LPS, in the absence or presence of Hb. After 4 h, TF procoagulant activity was determined with a plasma recalcification assay. TF activity was calculated from the turbidity generated in plasma (the mean from 6–8 replicate wells) based on a standard curve established with dilutions of rabbit brain thromboplastin (Baxter Corporation, Miami, FL, USA). The turbidity generated at 8 min by 1:100 diluted thromboplastin was defined as 1 TF arbitrary unit.

RESULTS

A series of LPS was obtained for the purpose of relating LPS biochemical structure with (1) LPS procoagulant biological activities, and (2) the ability of Hb to enhance these intrinsic LPS activities. In order to examine the role of the hydrophilic core and O chain saccharide on Hb enhancement of LPS activity, 4 *P. mirabilis* LPS were compared for LAL activities in the absence and presence of Hb. These LPS, assayed for relative biological activity in the presence of ααHb, all demonstrated enhanced LAL activities (Fig. 1A). Similar extents of enhancement also were shown for ααHbCO and HbA_0 (data not shown). For each LPS, enhancement by HSA (another known LPS binding protein[31]) was observed, although the levels of enhancement by Hb were 2- to 3-fold greater than by HSA (data not shown). None of the Hb preparations or HSA activated LAL in the absence of LPS. Because prominent enhancement by ααHb was observed with the deep rough mutant R45 LPS (containing only lipid A, Kdo and Ara4N), our results indicated that the Hb enhancement effect was a feature of the hydrophobic part of the LPS molecule, and did not require additional hydrophilic saccharide components.

The 4 *P. mirabilis* LPS had different intrinsic biological abilities to activate LAL, as shown in Figure 1A. The parent strain S1959 LPS was 1.3-fold more reactive than R110 LPS, which contains the complete core oligosaccharide, and 3.4 times more reactive than the deep rough mutant R45 LPS. These relative activities suggested that the presence of core oligosaccharide and O-specific polysaccharide moieties increased LAL reactivity. 03 LPS was 2.8 times more reactive than S1959. Since the O-specific polysaccharide chains of the 2 smooth strain LPS, S1959 and 03, differ significantly in their chemical structures, our data also suggested that carbohydrate chemical structure can influence LAL activity.

To ascertain whether enhancement of *Proteus* LPS biological activity by Hb would be observed for another LPS-dependent procoagulant activity, we utilized an assay for tissue factor (TF) production by human endothelial cells. ααHb enhanced LPS-stimulated TF production by the 2 rough *P. mirabilis* mutant LPS, R45 and R110, and by the smooth LPS S1959, but not by the smooth LPS 03 (Fig. 1B). Endothelial cells alone or in the presence of ααHb without LPS did not produce measurable TF (data not shown). When S1959 and its mutant LPS R45 and R110 were compared, there was an inverse relationship between relative intrinsic TF production and enhanceability by Hb. Prominent enhancement with R45 LPS provided additional evidence that the Hb enhancement effect was a feature of the lipid A portion of LPS. In both the LAL and TF assays, the activity of 03 LPS was affected the least by ααHb.

In order to examine the role of lipid A structures in the Hb enhancement effect, biological activities of *S. minnesota* R 595 LPS and chemically modified LPS partial structures were compared in the presence and absence of Hb. Intrinsic biological abilities of these *S. minnesota* LPS to activate LAL, and their respective extents of enhancement by Hb, are shown in Figure 2A. Only the parent 595 LPS demonstrated enhancement of LAL activity by Hb. Similar extents of enhancement also were shown for ααHbCO, HbA_0 and HSA (data not shown). Hydrolysis of even a single fatty acid from R 595 LPS, forming OH37 LPS, completely destroyed the enhancement potential of this LPS. Enhancement of 595 LPS biological activity by ααHb also was observed in the assay for LPS-induced endothelial cell TF (Fig. 2B). These results confirmed the intrinsic Hb enhanceability of rough 595 LPS observed with LAL activation and the observation that fatty acid hydrolysis resulted in destruction of the Hb enhancement potential of LPS. Interestingly, stimulation of TF by lipid A was prominently enhanced by Hb, whereas LAL activation was not.

Comparison of R 595 intrinsic LAL activities provided data linking LPS structure with LAL biological activity. R 595 LPS, which was relatively insoluble, had modest biological activity (Fig. 2A). Partially deacylated OH37 LPS, which was relatively soluble, readily activated LAL compared to the parent 595 LPS. Lipid A also demonstrated high biological activity. MPL, which was the most insoluble, and OH56 LPS poorly activated LAL. These results indicated that removal of a single 3-hydroxytetradecanoyl acid residue

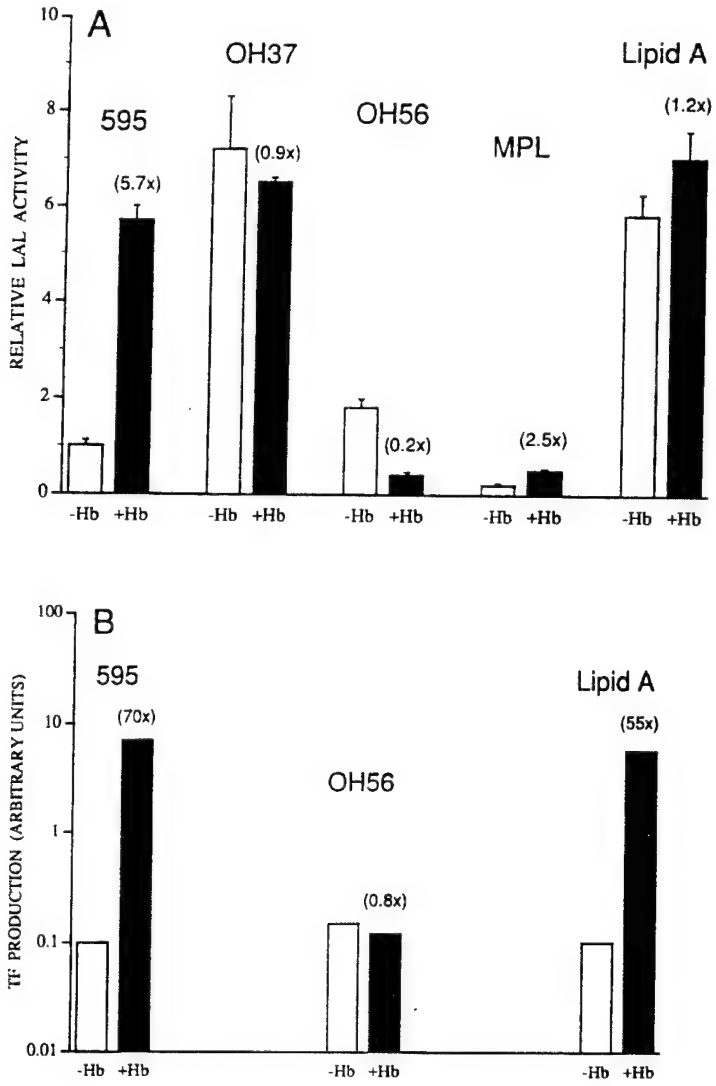

Fig. 2 — Enhancement by Hb of the activation of LAL and production of endothelial cell tissue factor by *Salmonella* LPS. (**A**) LAL reactivities of LPS (500 ng/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (1 mg/ml) were determined with the chromogenic LAL assay. To determine relative LAL activities, a standard curve of parent *S. minnesota* R 595 LPS was prepared, which related absorbance to LPS concentration. Using this standard curve, the absorbance for each sample (LPS alone or LPS-Hb) was converted into the equivalent R 595 LPS concentration. 500 ng/ml R 595 LPS was assigned a relative LAL activity of 1. The fold increase in LAL activity of each LPS, induced by Hb, is indicated in parentheses. Samples were assayed with 8 replicates, and results are expressed as the mean ± 1 SD. (**B**) Cultured human umbilical vein endothelial cells were incubated with *Salmonella* LPS (10 μg/ml) in the absence (–Hb) or presence (+Hb) of αα cross-linked Hb (10 mg/ml). TF activities then were determined with a plasma recalcification assay. The fold increase in TF induced by Hb for each LPS is indicated in parentheses. The means of 4 wells are presented.

from the reducing glucosamine of the lipid A moiety of R 595 LPS, or removal of the Kdo molecule from the core region, generated modified LPS molecules which could more efficiently interact with LAL, whereas further deacylation of the R 595 LPS to OH56 LPS resulted in a modified LPS with biological activity similar to that of the parent LPS. Therefore, the core Kdo and at least one of the fatty acid residues of lipid A apparently are not crucial for LAL activation. There was a dramatic loss of LAL reactivity after partial dephosphorylation of LPS to generate MPL, indicating that the phosphate group of lipid A was critical for LAL reactivity or that biologic activity was dependent upon adequate solubility,

Many of the LPS preparations studied had poor aqueous solubility and were visually turbid (especially *S. minnesota* 595 LPS, lipid A and MPL, and *P. mirabilis* R110). Hb enhancement of LPS biological activity was a prominent feature of some of these LPS and partial structures, suggesting that a possible mechanism for the Hb enhancement effect was via increased LPS solubility. Therefore, we compared turbidity and the LAL biological activity of these LPS in the absence and presence of Hb. With increasing concentrations of ααHb, *P. mirabilis* R110 and *S. minnesota* 595 LPS each demonstrated a concomitant progressive decrease in turbidity and increase in LAL biological activity (Fig. 3). A similar but smaller change in turbidity, and an increase in LPS biological activity, were observed with lipid A. MPL turbidity was shown to increase rather

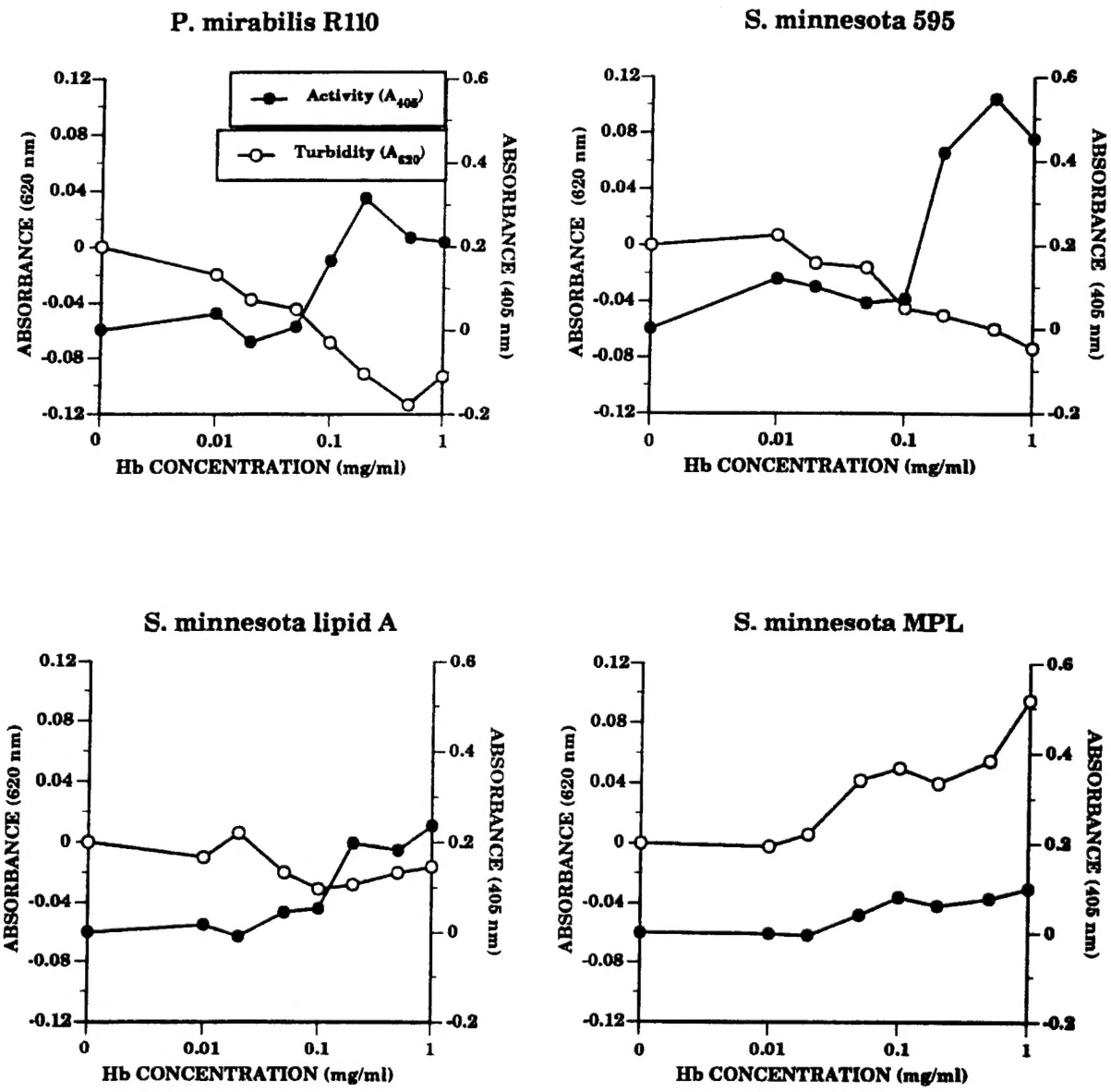

Fig. 3 — Turbidity and biologic activities of LPS in the absence and presence of Hb. Various concentrations of αα cross-linked Hb (from 0.01 to 1.0 mg/ml) were added to LPS (final concentration, 1 mg/ml) in microtiter plate wells and absorbances were measured at 620 nm. The turbidity of each LPS (absorbance at 620 nm) in the absence of Hb has been designated as 0, and the change in absorbance induced by Hb is shown. Absorbances due to Hb have been subtracted. Actual baseline LPS absorbances were as follows: *P. mirabilis* R110, 0.21; *S. minnesota* R 595, 0.12; *S. minnesota* lipid A, 0.61; and *S. minnesota* MPL, 0.65. LAL then was added to each well and chromogenic activities determined at 405 nm.

than decrease with addition of ααHb, and biological activity was unchanged. In a control experiment using the same LPS, HSA demonstrated no effect on LPS turbidity (data not shown). In this experiment, HSA enhanced the biological activity of R110 and had little effect on the other LPS.

In order to further establish the generalized nature of the Hb enhancement effect, we studied the effect of ααHb on biological activities of several other LPS, including LPS from different bacterial species. Prominent, and identical, extents of enhancement by both ααHb and ααHbCO in the LAL assay were shown with 3 defined salts of *E. coli* O26:B6 (smooth LPS), i.e. the calcium, sodium and triethylamine forms, suggesting that the specific cations bound to LPS did not influence the Hb enhancement process. *E. coli* O26:B6 LPS-induced endothelial cell TF also was enhanced by ααHb (mean of 13-fold enhancement in 7 experiments, range 8- to 26-fold enhancement). Finally, enhancement of LPS biological activity was demonstrated with a smooth *Salmonella* LPS (*S. abortus equi*) and a rough *E. coli* LPS (Re F515), but was not observed with non-toxic *R. spheroides*, *R. capsulatus* and *Rh. viridis* LPS (data for the above not shown).

To compare the enhancement ability of Hb with other plasma proteins, LAL activation by *S. minnesota* R 595 LPS was determined in the absence or presence of Hb and several other previously identified LPS binding protein. Because of limited availability of LPS binding protein, the comparison of Hb and LPS binding

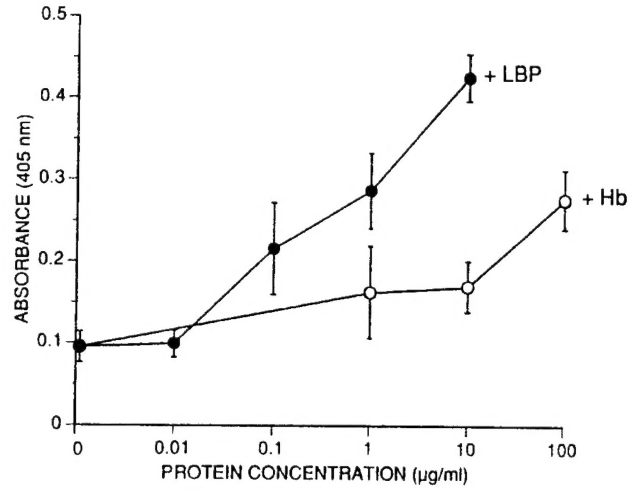

Fig. 4 — Comparison of Hb and LBP for ability to enhance LPS activation of LAL. *S. minnesota* R 595 LPS (10 pg/ml), in the absence or presence of αα cross-linked Hb in concentrations ranging from 1 to 100 µg/ml) or LBP (lipopolysaccharide binding protein) in concentrations ranging from 0.01 to 10 µg/ml), was assayed with the chromogenic LAL test using undiluted LAL. Samples were assayed in triplicate, and results are expressed as the mean ± 1 SD.

protein was performed using undiluted LAL, as described in Methods. In this experiment, the biological activity of 10 pg/ml LPS was clearly initially increased in the presence of 0.1 µg/ml LBP or 1–10 µg/ml ααHb (Fig. 4). Throughout almost the entire range of ααHb concentrations tested for enhancement of 595 LPS biological activity, equivalent enhancement was produced by LBP at a much lower protein concentration.

Comparisons also were made between HbA_0 and HSA, IgG or transferrin using the standard chromogenic assay (Fig. 5). HbA_0 and albumin enhanced *S. minnesota* R 595 LPS LAL reactivity in a dose-dependent manner, although the enhancement effect of HbA_0 was detectable at 0.03 mg/ml, whereas equivalent enhancement of 595 LPS by albumin was only observed at > 0.25 mg/ml protein. In contrast to LPS enhancement by HbA_0 and albumin, neither IgG nor transferrin had any demonstrable effect on 595 LPS LAL reactivity. Similar results were obtained using ααHb in comparison with albumin, IgG and transferrin (data not shown). Therefore, of the 5 human proteins studied, LBP and Hb were effective at low concentrations (≤ 0.03 mg/ml) in enhancing LAL activation by LPS, whereas albumin, IgG and transferrin had little or no such capability at comparable concentrations (compare Figs 4 and 5). The lack of effect of IgG on LAL activation by LPS is in contrast to our previous study which demonstrated inhibition by IgG.[16] However, subsequent investigation has demonstrated that the inhibitory activity detected in the previously studied preparation of IgG was attributable to maltose used as a stabilizer rather than the IgG per se (personal observation).

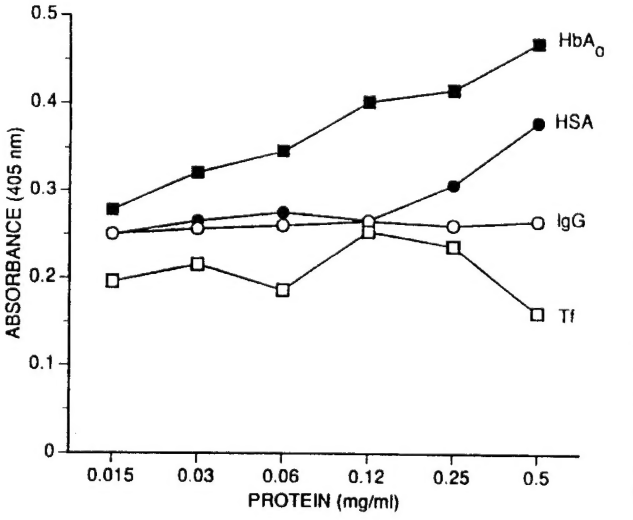

Fig. 5 — Influence of plasma proteins on the ability of *S. minnesota* R 595 LPS to activate LAL. *S. minnesota* R 595 LPS (30 ng/ml) was incubated for 5 min at 37°C with native HbA_0, HSA, human IgG or human transferrin (Tf) (each at concentrations from 0.015 to 0.5 mg/ml), and chromogenic LAL assays were performed. Absorbance at 405 nm for LPS alone was 0.24. Samples were assayed in duplicate, and mean values are presented.

Since IgG did not enhance the LAL reaction, it was possible to investigate the potential of this known LPS binding protein to inhibit LPS enhancement by Hb. Therefore, we studied whether the presence of IgG would prevent enhancement of LPS-induced LAL activation by Hb. *S. minnesota* R 595 LPS was preincubated with either IgG or HbA_0, followed by subsequent incubation with the other protein. Addition of IgG to a preincubated mixture of LPS and HbA_0 slightly decreased Hb-induced enhancement at the lower concentrations of LPS studied, but had no effect at higher LPS concentrations (data not shown). Addition of HbA_0 to a preincubated mixture of LPS and IgG similarly demonstrated partial inhibition of the Hb effect at the lower LPS concentrations, but no inhibition at higher LPS concentrations. These results are consistent with competition between Hb and IgG for LPS, but with Hb demonstrating the greater affinity.

DISCUSSION

LAL reactivities of our battery of LPS were examined in the presence of Hb in order to define the constituents of the LPS molecule required for interaction with Hb. ααHb enhanced the biological activity of each of the *Proteus* LPS tested, including R45 LPS which contains only lipid A, Kdo and 4-amino-arabinose residues.[19,20] This suggested that the hydrophobic part of LPS, i.e. lipid A, was responsible for the interaction with Hb and the resultant increase of biological activity of LPS. ααHb also enhanced rough LPS-stimulated tissue factor production from endothelial cells, and thus provided further support for the conclusion that components of lipid A are required for interaction with Hb. Lipid A chemical moieties then were studied utilizing *S. minnesota* R 595 LPS and its chemically modified derivatives in the presence of Hb. Whereas intact R 595 LPS

demonstrated prominent enhancement of its biological activity by ααHb, singly deacylated OH37 LPS and multiply deacylated OH56 LPS failed to be enhanced in the LAL assay. Similarly, OH56 LPS failed to be enhanced in the endothelial cell tissue factor assay. These results suggested that the binding process between Hb and LPS, that leads to enhancement, may involve an important interaction of this protein with the ester-linked fatty acyl residues of lipid A. Our studies demonstrated a similar necessity for lipid A fatty acids for the increase in LPS biological activity induced by human albumin, an avid fatty acid binding protein. Enhancement of MPL (lacking the phosphate residue of the reducing glucosamine) or lipid A (lacking the Kdo residues of the core) was substantially less than that of the parent R 595 LPS, but was not totally absent as for the deacylated LPS (Fig. 2). These results suggested that phosphate and Kdo residues are less important in the enhancement process than the ester-linked fatty acids.

In general, those LPS with poor solubility in water (i.e. *P. mirabilis* R45, *S. minnesota* R 595 and its MPL derivative) were more effectively enhanced by Hb than those with better solubility (i.e. *S. minnesota* R 595 derivatives OH37 and OH56). This suggested that Hb has a more important detergent-like, disaggregating effect on those LPS with poor initial solubility. This conclusion is supported by our previous observations that Hb decreases the apparent molecular weight of LPS.[15] In further support of this potential mechanism for Hb enhancement of LPS biological activity, we demonstrated that ααHb decreased the turbidity (i.e. increased the solubility) of the poorly soluble LPS. Disaggregation of LPS micelles, resulting in enhanced LAL activity of LPS, similarly has been proposed as the reason for the increased potency of LPS in the presence of transferrin.[32] It is interesting that enhancement of LPS biological activity by albumin in our studies was not associated with decreased LPS turbidity, suggesting that Hb and albumin enhance LPS activity by different mechanisms.

In contrast to the effects of chemical modification of LPS on their LAL biological activities and the process of Hb enhancement, three distinct salt forms of *E. coli* O26 LPS (Na, Ca and triethylamine) had similar LAL reactivities and demonstrated pronounced and identical enhancement by ααHb. These results are in agreement with previous observations that *Limulus* gelation activities were similar for a variety of electrodialyzed defined salts of *S. abortus equi* LPS.[33]

Equivalent extents of Hb enhancement of LPS activity were observed with native HbA_0 as with ααHb, demonstrating that this was an intrinsic property of Hb. Enhancement of bioavailability of LPS with cross-linked Hb indicated that dissociation of the Hb tetramer was not involved in this effect. Because equivalent enhancement results also were obtained with ααHbCO, the mechanism of the enhancement effect did not require the production of methemoglobin, and enhancement was not a result Hb oxidation and denaturation. However, we have observed that LPS in high concentration (0.5–1 mg/ml), is capable of facilitating Hb denaturation (unpublished observations).

Our comparisons of the relative LAL activities of *Proteus* and *Salmonella* LPS specifically demonstrated the contribution of the Kdo residues of the core, and the fatty acyl and phosphoryl residues of lipid A, to LPS intrinsic biological activity. The alterations in LAL potencies of chemically modified 595 LPS may have resulted from changes in solubility in aqueous solutions of the modified LPS as well as the removal of residues important for LAL activation. In addition, heterogeneity of both native and chemically degraded *S. minnesota* 595 LPS may also influence LAL reactivity. These observations are consistent with previous studies concluding that *Salmonella* and *Escherichia* lipid A require at least one phosphate residue and two fatty acyl chains for activation of LAL,[34–36] and that chemically synthesized MPL[37] or highly purified *E. coli* MPL[34] have reduced LAL potency. Interestingly, 3-O-deacylated LPS such as the *S. minnesota* OH37 LPS we prepared is known to possess a large and energetically unfavorable cavity in the interior part of the LPS molecule which results in a conformational reorganization of lipid A,[25] and in our experiments this chemical modification resulted in increased biological activity in the LAL assay. In contrast, similar 3-O-deacylation of *E. coli* J5 and *S. typhimurium* LPS has been shown to result in LPS with unaltered LAL activity and mitogenicity, and with attenuated rabbit pyrogenicity and decreased chick embryo and mouse lethality,[25] and the removal of secondary acyl chains from the lipid A moiety of LPS by leukocyte acyloxyacyl hydrolase has been reported previously to reduce LPS LAL potency.[38] It is not known whether these differences are related to the chemical composition of the LPS tested or to differences in the biological assays utilized. The enhancement of LPS biological activity by Hb also requires these lipid A moieties and in addition, Kdo residues, and likely represents a mechanism that involves the disaggregation and solubilization of LPS by Hb. Interestingly, Hb did not enhance LAL activity of non-enterobacterial, non-toxic *Rhodobacter* and *Rhodopseudomonas* LPS. This may indicate that a 'toxic conformation'[39] of LPS, not present in non-toxic LPS,[40] is required for Hb enhancement. It is likely that the phenomenon of Hb enhancement of LPS biological activity has physiologic and clinical relevance because of the difficulty in production of Hb without substantial endotoxin contamination,[16] the likelihood that Hb would be transfused into trauma patients with concomitant endotoxemia, and the potential for hemoglobinemia (secondary to intravascular hemolysis during sepsis) to augment the deleterious effects of endotoxemia.

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